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THE EFFECTS OF ESTROGEN AND SERMs ON BONE CELL VIABILITY

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A Thesis submitted for the degree of Doctor of Philosophy

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Thesis Abstract

Estrogen withdrawal due to either menopause or ovariectomy has previously been shown to promote bone loss and to be associated with the apoptotic death of osteocytes *in vivo*. The presence of viable osteocytes in bone has been hypothesized to be an important parameter in maintaining bone quality while osteocyte death by apoptosis during estrogen deficiency has been associated with loss of bone strength. Estrogen (17β-estradiol) administration, as part of hormone replacement therapy (HRT), has previously been shown to exert positive effects on the maintenance of osteocytes through the inhibition of their apoptotic cell death. Although the beneficial role of other anti-resorptive agents such as the Selective Estrogen Receptor Modulators (SERMs) in preventing bone loss in postmenopausal osteoporosis are well established, the effects of SERMs on the maintenance of the osteocytic population in vivo are less clear. Work in this thesis has investigated whether the SERM LY 117018, which is a Raloxifene analogue, can mimic the osteocyte-sparing effect of 17β-estradiol in a rat model of ovariectomy.

Abrupt estrogen withdrawal was shown to increase osteocyte apoptosis in rat bone, while the ovariectomy-induced stimulation of osteocyte apoptosis in rats was shown to be reversed following administration of the LY 117018 SERM in a similar way to the known effect of 17β-estradiol replacement. These data point to the potential benefits of SERMs on bone viability in estrogen-depleted rats. However, the molecular mechanism by which 17β-estradiol and the LY 117018 SERM maintain the osteocytic population has to date not been well characterised.

In vitro studies in this thesis indicated that 17β-estradiol, raloxifene and LY 117018 suppressed the pro-apoptotic stimuli induced in response to H_2O_2 in osteocyte cultures possibly by exerting direct anti-oxidant properties related to their chemical structure. These data introduced a novel mechanism of action for 17β-estradiol, raloxifene and LY 117018, as antioxidants, in the protection of the osteocytic population increasing the knowledge available on their activity in bone. In addition, these data suggested that the

effects of SERMS on preventing osteocyte apoptosis will in future help to determine their effectiveness and interest for the clinical development of estrogen replacement compounds with activities consistent with the maintenance of both bone mass and bone quality.

Finally, the effect of mechanical loading on the maintenance of the osteocytic population was investigated in human cancellous bone explants *ex vivo* in the presence or absence of 17β-estradiol. Results suggested that mechanical stimulation or 17β-estradiol alone increased the Bone Formation Rate (BFR/BS) compared to the unloaded conditions after 27 days in culture. Furthermore, application of mechanical loading at physiological levels improved osteocyte viability and significantly reduced osteocyte apoptosis relative to that seen under disuse conditions.

In this study identification of anti-apoptotic effects of SERMs on osteocytes *in vivo* and *in vitro* highlighted an important bioactivity that may explain part of the action of SERMS *in vivo*. Further characterization of the anti-apoptotic effects of 17β-estradiol and SERMs *in vitro* indicated direct antioxidant capabilities pointing to the possibility that SERMs mimic not only the bone-sparing activity of 17β-estradiol but its anti-oxidant nature as well.

Declaration

I hereby declare that this thesis has been composed by myself and is the result of my own work. I was assisted through the donation of cellular material, as clearly indicated throughout the text of the thesis. This work has neither been submitted for any other degree.

Christina Huber
January 2007

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I am also grateful to Prof. Philippa Saunders (University of Edinburgh, UK) for the provision of the HEK 293 cell line.

To my cherished friend Yolanta

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Abbreviations

AD	Alzheimer's disease
ALP	alkaline phosphatase
ANOVA	analysis of variance
ASBMR	american society of bone and mineral research
ATP	adenine triphosphate
BFR	bone formation rate
BMD	bone mineral density
BMPs	bone morhogenetic proteins
BMSC	bone marrow stromal cells
BMUs	basic multicellular units
bp	base pairs
BS	bone surface
BV	bone volume
CAD	caspase-activated Dnase
C-	carbon
^{o}C	degrees Celcius
Ca^{+2}	calcium ion
Caspases	cysteine aspartate proteases
CAT	catalase
Cbfa1	core-binding factor a -1
CFU-GM	colony forming unit of the granulocyte-macrophage series
cm	centimeter
CO_2	carbon dioxide
Cx	connexin
2D	2-dimensional
3D	3-dimensional
DAPI	4', 6-Diamidino-2-phenylindole
dATP	2'-deoxy-adenosine 5'-triphosphate
dCTP	2'-deoxy-cytidine 5'-triphosphate
DERKO	double estrogen receptor knock out

DFF45	DNA fragmentation factor 45
dGTP	2'-deoxy-guanosine 5'-triphosphate
DHEA	dehydroepiandrosterone
DIG	digoxigenin
dLS	double labelled surface
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	deoxynucleoside triphosphate
dUTP	2'-deoxy-uracil 5'-triphosphate
E_1	estrone
E_2	17β-estradiol (estrogen)
E_3	estriol
EBPs	estrogen binding proteins
ecNOs	endothelial cell nitric oxide synthase
EDTA	ethylenediaminetetraactic acid
EGF	endothelial growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
EREs	estrogen response elements
ERK	extracellular signal regulated kinase
αERKO	estrogen receptor alpha knock out
βERKO	estrogen receptor beta knock out
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
FasL	Fas ligand
FBS	fetal bovine serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GLAST	glutamate and aspartate transporter
GNRH	gonadotrophin-releasing hormone

L-Glu	L-glutamine
GnRH	gonadotrophin-releasing hormone analogs
GPx	glutathione peroxidase
GR	glucocorticoid receptor
GRP30	G-protein coupled receptor 30
GSH	Glutathione
H-	hydrogen atom
H^+	hydrogen proton
HBSS	Hank's balanced salt solution
H_2DCF-DA	2',7'-dichlorodihydrofluorescein-diacetate
H_2O	water
H_2O_2	hydrogen peroxide
HeLa cells	Henrietta Lacks cells
HRT	hormone replacement therapy
Hz	Hertz
ICAD	inhibitor of caspase activated deoxyribonuclease
IFN-γ	interferon-gamma
IGF	insulin-like growth factor
IGF-1R	IGF-1 receptor
Ihh	indian hedgehog
IL-1	interleukin-1
IL-8	interleukin-8
Ir.L.t	interlabel time
Ir.L.Th	interlabel thickness
kg	kilogram
KO	knock out
L	litre
LDH	lactate dehydrogenase
LDL	low density lipoproteins
LRP5	low density lipoprotein receptor-related protein 5
M	molar
MAPK	mitogen-activated protein kinase

MAR	mineral apposition rate
MCSF	macrophage colony stimulating factor 1
MEK1/2	MAPK/ERK kinase
MEM	minimum essential medium Eagle
α-MEM	α-modified minimum essential medium
mg	milligram
Mg	magnesium
ml	millilitre
mm	millimetre
mmol	millimole
mM	millimolar
mRNA	messenger ribonucleic acid
MS	mineralising surface
NAD	α-nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NBT	nitroblue tetrazolium
NCS	newborn calf serum
ng	nanogram
nm	nanometer
NO	nitric oxide
NT	nick translation
O-	oxygen atom
$O_2^{\cdot -}$	superoxide
OF45	osteoblast/osteocyte factor 45
-OH	hydroxyl group
OH^-	hydroxyl radical
OPG	osteoprotegerin
OPN	osteopontin
OVX	ovariectomy
P/S	penicillin/streptomycin
PARP	poly(ADP-ribose) polymerase
PBS	phosphate-buffered saline

PGE_2	prostaglandin E_2
PHEX	Phosphate-regulating gene with homology to endopeptidases on the X chromosome
PI	propidium iodide
PI3-Kinase	phosphoinositol 3-kinase
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related protein
RANK	receptor for activation of nuclear factor kB
RANKL	receptor activator of NF-kB ligand
RO_2	peroxyl radical
ROS	reactive oxygen species
RT	room temperature
S.E.	standard error of the mean
SERMs	selective estrogen receptor modulators
sLS	single labelled surface
SOD	superoxide dismutase
SOST	sclerostin gene
TESPA	3-aminopropylnethoxy-silane
TGF-a	transforming growth factor-alpha
TGF-β	transforming growth factor-beta
TNF-a	tumor necrosis factor-alpha
TRAP	tartrate resistant acid phosphatase
VDR	vitamin D receptor
VEH	vehicle
μM	micromolar

SECTION 1

Introduction

CHAPTER 1

The effects of 17β-estradiol and Selective Estrogen Receptor Modulators (SERMs) on bone.

1.1 Introduction

Bone is a rigid form of connective tissue that supports locomotion as a site of muscle attachment, provides protection for vital organs, nurtures the bone marrow and stores ions such as calcium and phosphate that participate in serum homeostasis (Felsenfeld et al. 1999, Rasmussen et al. 1971, Baron 1996). Bone is a highly diverse and dynamic tissue that goes through continuous modelling and remodelling cycles during life in order to sense structural and mechanical requirements, adapt its architecture, accordingly and repair any damage occurring as a result of everyday use (Wolf 1892, Turner 1992, Parfitt 1994).

The processes of bone modelling and remodelling are controlled by a number of hormones, growth factors and cytokines (Rodan 1992). Steroid hormones, such as estrogens, have been shown to influence bone cells in order to maintain a balance between bone resorption and bone formation. Estrogen deficiency has been shown to result in bone loss due to increased bone resorption and impaired bone formation (Raisz 2005). Imbalances between bone resorption and formation may lead to abnormal turnover cycles, characterised by increased resorptive activity, that often lead to bone diseases such as osteoporosis. Osteocytes, which have been proposed to target the remodelling process by undergoing apoptosis (Noble et al. 1997, Verborgt et al. 2000), have been shown to be negatively affected by estrogen deficiency (Tomkinson et al., 1997; 1998, Kousteni et al. 2001). The aim of this thesis was to investigate the effects of estrogen and SERMs on osteocyte viability and apoptosis induced by estrogen deficiency in vivo and by oxidative stress in vitro.

This chapter outlines the organisation of bone, describes osteocytes and the apoptotic bone cell death and summarises the effects of estrogen and SERMs on bone.

1.2 Bone organisation

Bone consists of hydroxyapatite crystals, non-collagenous proteins and collagen fibres which are organised in parallel or concentric layers giving bone its lamellar structure (Baron 1996). Bones can be categorised into flat bones such as bones in the skull and the ilium and long bones, such as the tibia and the femur.

Long bones (**Figure 1.1**) consist of cortical (**Figure 1.2A**) and trabecular bone (**Figure 1.2B**) (Carter and Orr 1992, Parfitt 1994, Baron 1996) both of which contribute to their ability to function as the load-bearing parts of the skeleton. Cortical bone is a dense structure that forms the outer shell of the long bones and encloses the medullary cavity. The construction of the medullary cavity in long bones achieves stiffness and increases resistance to bending (Ruff 1988) and deformation, characteristics necessary for enabling weight bearing and mobility. Cortical bone consists of osteons (**Figure 1.2A**) oriented in the direction of maximal stress (Eihorn 1996). Each osteon, which is composed of a blood vessel enclosed in a Harvesian canal, is surrounded by concentric lamellae of collagen fibres (**Figure 1.2A**) and interstitial bone (areas of cortical bone surrounded by cement lines that do not contain an osteon). The surface of the cortical bone which faces the bone marrow is called the endosteum while the outer surface facing the soft tissue is called the periosteum (**Figure 1.1**).

Trabecular or cancellous bone is a highly porous structure located mainly in the vertebrae and the metaphyses of the long bones. It has a spongy appearance (**Figure 1.2B**) and consists of an inner network of interconnecting trabecular plates oriented in such a way in order to resist mechanical loading (Einhorn 1996, Weiner 1999, Sikavitsas et al. 2001). Due to its spongy appearance, trabecular bone contributes to more than 61% of the total bone surface area. The role of the trabecular bone is to be flexible (Turner 2002) in order to absorb more energy imparted during mechanical loading. Trabecular bone is capable of tolerating deformation; returning to its initial conformation before structural damage and distributing mechanical loads from the articular cartilage to the cortical shaft (Keaveny and Yeh 2002).

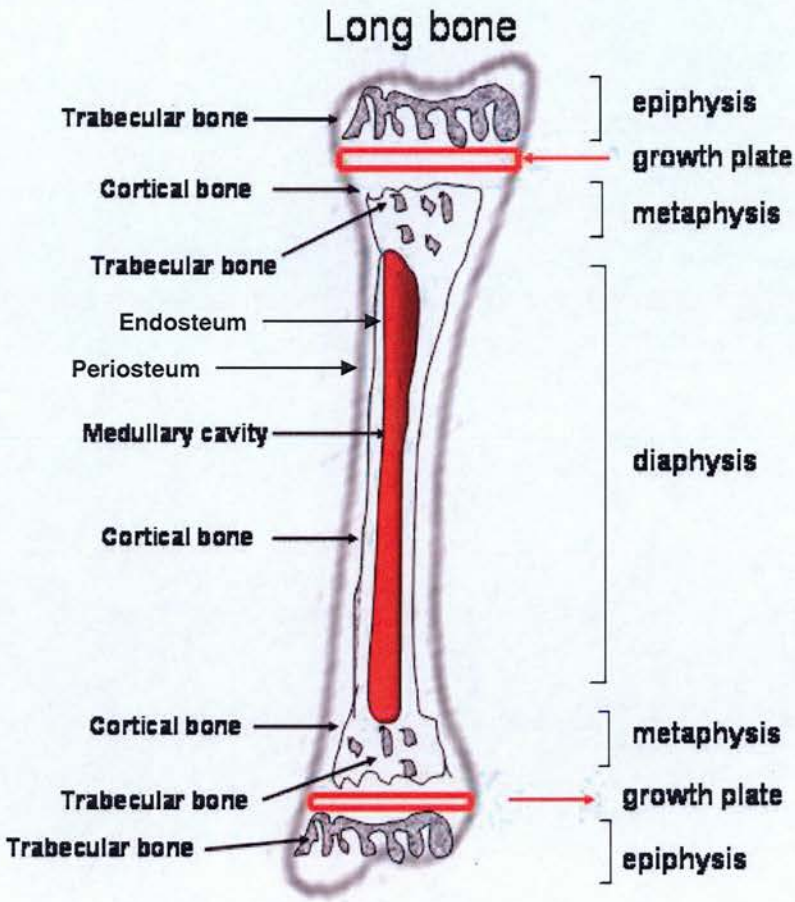
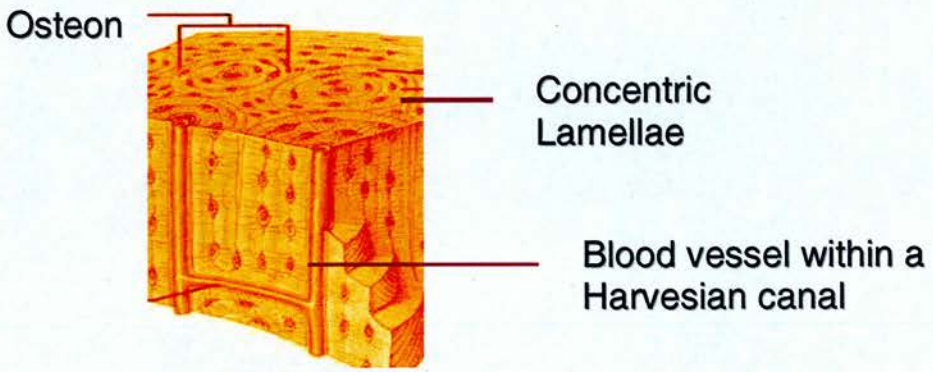


Figure 1.1. Schematic diagram of a long bone. Either end of a long bone consists of the epiphysis, characterised by internal cancellous structure, while the central region known as the diaphysis consists mainly of cortical bone. Diaphysis and epiphyses are separated by the metaphysis which includes both trabecular and cortical bone and is found below a zone of cartilage in which bone growth takes place known as the growth plate.

A. Compact bone



B. Trabecular bone

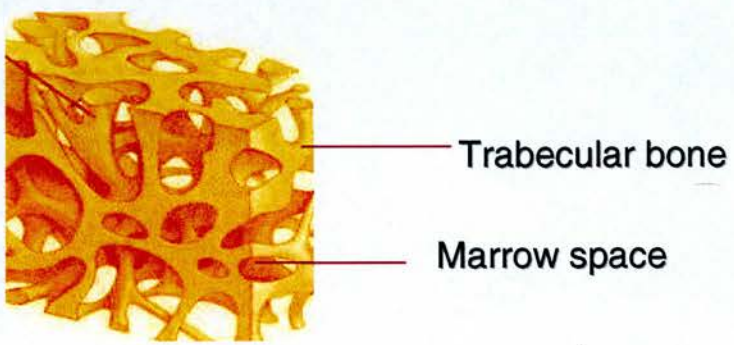


Figure 1.2. Schematic representation of (A) compact and (B) trabecular bone structure.

1.3 Modelling

During growth, skeletal architecture is established and controlled by the process of bone modelling. Modelling can be distinguished into; the intramembranous ossification process responsible for the formation of flat bones and the thickening of the long bones, and the endochondral ossification which forms the long bones. During intramembranous ossification, mesenchymal cells differentiate into osteoblasts (primary ossification centre) which synthesize and secrete the woven bone. Woven bone, which is characterised by irregular calcification and orientation of the collagen fibres and by high osteocyte cell density, is eventually replaced by the more structurally organised lamellar bone (Turner 1992, Weiner and Wagner 1998, Sikavitsas 2001).

Endochondral ossification (**Figure 1.3**) is a more complicated process that involves the differentiation of mesenchymal stem cells initially into chondroblasts, which form a cartilaginous template of the future bone and then differentiate into chondrocytes that eventually form the growth plate (secondary ossification centre) and bring about longitudinal (interstitial) growth (Parfitt 1976, Carter et al 1996). As chondroblasts continue to proliferate and secrete the cartilaginous matrix (interstitial growth), chondrocytes move further away from the proliferative zone and become hypertrophic. Hypertrophic chondrocytes initiate the mineralization process of the cartilaginous matrix (primary ossification centre) and eventually undergo apoptosis. This process is regulated by factors such as parathyroid hormone-related protein (PTHrP), Indian hedgehog (Ihh), and bone morphogenetic proteins (BMPs) (Strewler 2001). Osteoclasts then begin to resorb the calcified matrix, while osteoblasts, recruited to the primary ossification centre by blood vessels, use the calcified matrix as a template to form the woven bone on top of the resorbed areas, known as the primary spongiosum. The woven bone, located lower in the growth plate, will be further remodelled to form the lamellar bone, known as the secondary spongiosum (Boyde 1980, Parfitt 1994, Weiner and Wagner 1998, Baron 1996).

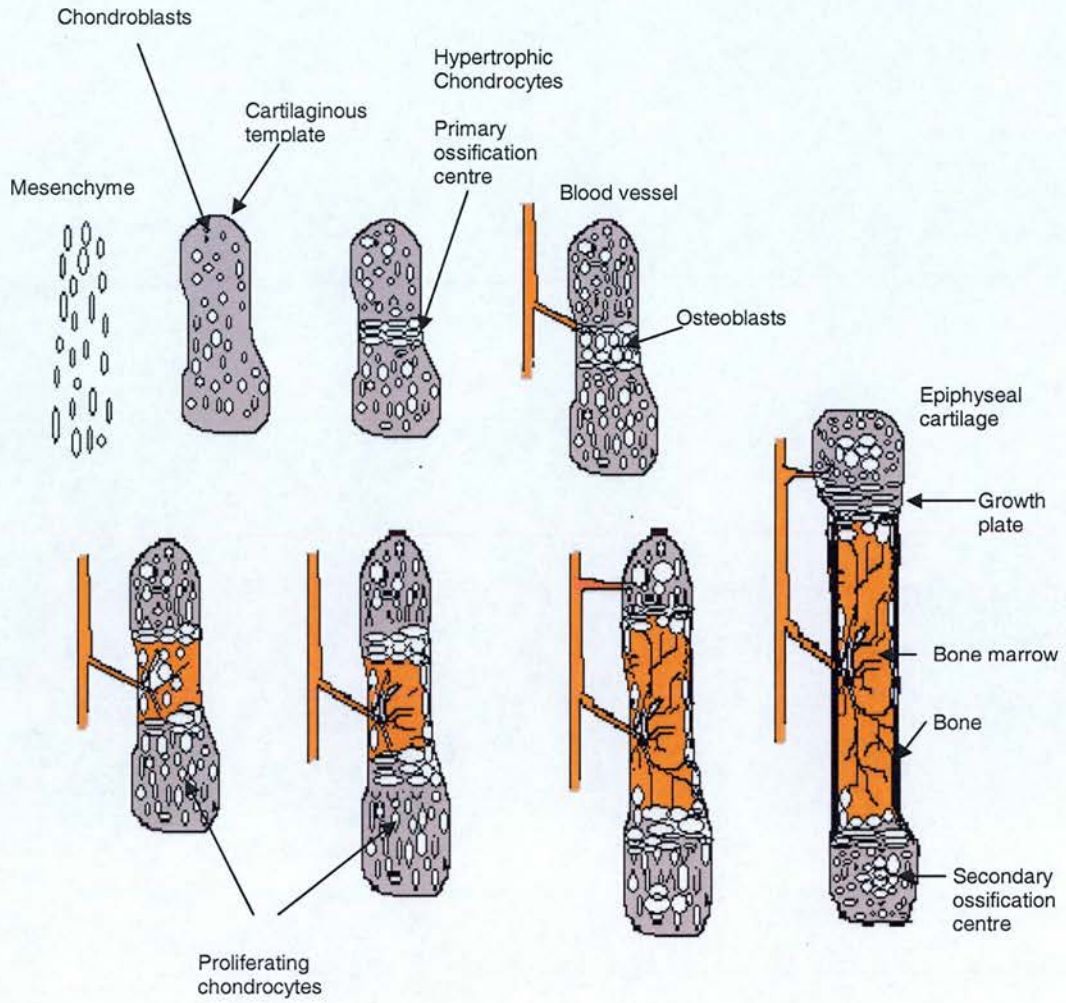


Figure 1.3. Endochondral ossification. During endochondral ossification mesenchymal stem cells differentiate into chondrocytes which produce a cartilaginous matrix that will be used as a template for the production of the future bone by the osteoblasts.

1.4 Bone remodelling

The bone remodelling or bone turnover process is a complex process occurring in both trabecular and cortical bone by which old or damaged bone is replaced by new bone (Mundy 1995). Bone remodelling is essential to bone in order to maintain skeletal architecture during the adult life, to repair microdamage in response to the mechanical requirements (Turner 1992, Parfitt 1994, Burr et al. 1985) and to regulate mineral homeostasis through the interaction of hormones, growth factors and cytokines with osteoblasts and osteoclasts.

In endochondral and trabecular bone remodelling the source of osteoblasts and osteoclasts is the stromal and haematopoietic precursors in the marrow, respectively, whereas in the periosteal remodelling osteoblasts are derived from the periosteal mesenchymal cells and the osteoclasts from peripheral blood haematopoietic precursors (Rodan 1992, Prockop 1997, Teitelbaum 2000). Bone turnover was proposed to occur in discrete basic multicellular units (BMUs) (Frost 1969) and to follow a cycle of tightly-regulated events initiated by the retraction of osteoblasts from a particular site in response to appropriate-yet uncharacterised-stimuli. This event is followed by the activation and recruitment of osteoclasts to the particular site through signals produced by osteoblasts and stromal cells in order to carry out bone resorption; osteoblasts replace the resorbed bone by secreting the collagenous and the non-collagenous matrix (osteoid) that eventually becomes calcified returning that site to quiescent state. The fate of osteoblasts is to either line the surface of the new bone, to undergo apoptosis or to differentiate into osteocytes as they become encased in their own matrix production (Parfitt 1994, Parfitt 2003).

1.5 Mechanical loading and bone

Mechanical loading controls both the modelling and the remodelling processes, playing an important role in the development of bone structure and shape during growth and the maintenance of this shape in adult life, respectively (Rubin 1984, Rubin and Lanyon 1987, Carter 1996, Mosley et al. 1997).

The ability of bone to respond to mechanical loading is best summarised by the mechanostat theory proposed by Frost (Frost 1988) which describes the ability of bone to adapt its structure to suit its function. Bone continuously adjusts to mechanical loading by changing its mass, shape, or microarchitecture and responds differently depending on the magnitude of the strain. For example, reduction in mechanical strain due to disuse situations such as prolonged bed-rest, space flight and reduced physical activity in old age results in increased bone resorption and bone loss (Inman et al., 1999), whereas under physiological strains bone mass is maintained. High strains are associated with increased bone formation and increased bone mass, while excessive mechanical loading could result in bone loss.

Evidence has suggested that bone has the ability to repair microdamage accumulating as a result of everyday use (Parfitt 2001) by sensing changes in the distribution of strain within the bone microenvironment and targeting bone for removal by the osteoclasts (Burr and Martin 1993, Parfitt 2002). In addition, several studies have demonstrated that microdamage induced by loading on canine (Burr and Martin 1993) or rat bone (Verborgt et al. 2000, Noble et al. 2003) was targeted for removal by osteoclastic cells more frequently than would have been expected by chance alone (Burr et al. 1985, Mon and Burr 1993, Burr and Martin 1993, Burr et al. 2001, Parfitt et al. 2001) followed by increased remodelling activity. The mechanism directing the targeted activity of bone forming and bone resorbing cells to specific sites is still not known. However, the osteocyte has been proposed by many studies to participate in this mechanism (Kakizaki et al. 1971, Kenzora et al. 1978, Kamijou 1994, Mori et al. 1997, Marrotti et al. 1998).

1.6 Bone forming and resorbing cells

Osteoblasts are derived from bone marrow stromal cells or pluripotent mesenchymal stem cells, and carry out bone formation. (Ducy et al. 2000, Prockop 1997). Central in the regulation of osteoblast differention and formation is the Indian hedgehog and sonic hedgehog, the transcription factor core-binding 1 (Cbfa1) and the bone morphogenetic proteins (Yamagushi et al. 2000). Osteoblast formation and proliferation are also regulated by the action of hormones such as PTH, estrogen, vitamin D and glucocorticoids (Beresford et al. 1992; 1994, Manolagas et al. 2000, Kousteni et al. 2001, Strewler et al. 2001); as well as by the expression of genes such as TGF-β, FGF and IGF. These factors influence matrix production, secretion of matrix proteins and mineralization of bone by regulating the expression of genes such as collagen type I and fibronectin, alkaline phosphatase (ALP), osteopontin and osteocalcin (Puzas et al. 1996).

Osteoblast activity is also regulated by members of the Wnt signalling pathways, since gain or loss of function mutations in the receptor LRP5 induce high bone mass or osteoporosis-pseudoglioma syndrome, respectively (Boyden et al. 2002). High bone mass phenotypes also arise from mutations in the SOST gene that leads to sclerosteosis, which is also implicated in the Wnt signalling pathway. Following the deposition of matrix, active osteoblasts differentiate into lining cells, apoptose or become encased in their own matrix production and differentiate into osteocytes (Jilka et al. 1998, Karsdal et al. 2002).

Osteoclasts are polynuclear cells formed by the fusion of monocytes of the, colony forming unit of the granulocyte-macrophage (CFU-GM) series and are derived from haematopoietic stem cells in the bone marrow or peripheral blood. In order to carry out bone resorption, osteoclasts which have a half-life of about two weeks, migrate to a particular site; form a ruffled border and polarise onto the bone tissue; degrade the bone matrix and the hydroxyapatite crystals; remove the resorption products and finally undergo apoptosis (Hughes and Boyce 1989, Xing and Boyce 2005).

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1.7 Osteocytes

Osteocytes are terminally differentiated osteoblastic cells that reside in lacunae within the bone matrix (Aarden et al. 1994) and are the most abundant type of cell in bone, while their density has been observed to be inversely related to the size of the species (Mullender 1996) (**Table 1.1**). It has been suggested that 10-20% of osteoblasts differentiate into osteocytes (Aubin et al. 1996); however the signals that regulate this process are not fully understood yet. Osteocytes communicate with other osteocytes and other cells on the bone surface through dendritic processes that radiate in all directions and pass through small canals, the canaliculi, in bone (Palumbo et al. 1990, Aarden et al. 1994).

Due to their location within the mineralised bone matrix and the lack of antibodies directed against specific osteocytic markers, very few studies describing the characteristics of osteocytes in culture are available to date. Avian postmitotic osteocytic-like cells (Van der Plaas et al. 1994) have been isolated and purified based on the use of an antibody termed mAb OB7.3 (Nijweide and Mulder 1986, 1992) which is thought to be specific for the chicken PHEX endoprotease (Phosphate-regulating gene with homology to endopeptidases on X chromosome) (Westbroek et al. 2002), possibly forming part of the OF45 signalling pathway (MacDougall et al. 2001, Gowen et al. 2003). Studies have localised the OF45 mRNA on mature osteoblasts and osteocytes

throughout ossification in the skeleton of rats and mice (Petersen et al. 2000, Igarashi et al. 2002) suggesting that OF45 might be involved in the mineralization process and the differentiation of osteoblasts to an osteocytic phenotype. Another possible marker of the osteocytic phenotype is the E11 antigen, which has been proposed to regulate formation and maintenance of the osteocytic cellular processes (Wetterwald et al. 1996, Schulze et al. 1999).

Most of the osteocytic characteristics in vitro have been based on the use of the murine osteocytic-like cell line called MLO-Y4 (Kato et al. 1997). MLO-Y4 osteocyte-like cells were isolated from the long bones of transgenic mice in which expression of the SV40 large T-antigen oncogene was targeted to osteocytes under the control of the osteocalcin promoter (Kato et al. 1997). These cells were able to proliferate and secrete high amounts of osteocalcin. In addition, MLO-Y4 are also characterised by long dendritic processes, osteopontin and connexin 43 expression, low collagen type I, low alkaline phosphatase expression (Kato et al. 1997) and E11 expression (Bonewald et al. 2000) suggesting that these cells display osteocyte-like properties. Other cell lines include the MLO-D1, -D6, -A5 and –C2 osteocyte-like cells, which are proposed to represent various stages in the differentiation of osteoblasts to osteocytes (Kato et al. 2001), and are not very well characterised yet.

Several functions have been proposed for the osteocytes, including their involvement in the mineralization process due to their high osteocalcin expression (Ikeda et al. 1996) and also bone resorption known as osteocytic osteolysis, as suggested by the lacunar size in diseases and collagenase production (Shimizu et al. 1990, Fuller and Chambers 1995), although this property remains controversial (Boyde 1980, Marotti et al. 1990, Van der Plaas et al. 1994). Other studies have suggested that osteocytes provide an inhibitory signal to neighbouring osteoblasts that negatively regulates bone formation (Marotti et al. 1996). Recently, several groups have proposed this inhibitory signal to be the protein sclerostin (van Bezooijen et al. 2004; 2005, Poole et al. 2005), the product of the SOST gene responsible for sclerosteosis (Brunkow et al. 2001), which is localised on mature

osteocytes. Studies have suggested that sclerostin exerts pro-apoptotic effects on human osteoblastic cells possibly by binding to BMPs reducing their availability and therefore decreasing BMP signalling (Sutherland et al. 2004). However, research on this field is still ongoing and further studies are required to conclude that osteocytes negatively regulate bone formation.

In contrast to this theory come studies in which examination of the lacunar occupancy at different stages of the remodelling cycle revealed that forming as well as resorbing osteons contained higher osteocyte density and lacunar occupancy, compared to quiescent osteons (Power et al. 2002). In addition, there is evidence accumulating that the controlled death of osteocytes through apoptosis might be providing the signals that direct turnover, since apoptotic osteocytes have been observed in close association with resorbing areas (Noble et al. 1997, Verborgt et al. 2000) and most importantly prior to initiation of bone resorption (Noble et al. 2003). However, in order for the osteocytes to target remodelling to specific areas, they should be able to sense such a requirement.

The communicating network that osteocytes form as well as their location within the bone matrix have suggested that osteocytes might sense the amount of strain applied in the local environment (Lanyon 1993, Klein Nulend et al. 1995, Zhang et al. 1997, Noble et al. 2003) and communicate a metabolic signal to bone effector cells (Duncan and Turner 1995, Lean et al. 1996, Aarden et al. 1996, Klein Nulend et al. 1995, Yellowley et al. 2000). Examples of such load-induced responses in osteocytes, would be the production of prostaglandin E_2 (PGE_2) which has been shown to induce osteoclast maturation and osteoblast proliferation and differentiation (Suda et al. 1995, Samoto et al. 2003); release of NO from ecNOs, the expression of which has been suggested to regulate the direction of resorption and pit size formation (Loveridge et al. 2002, Burger et al. 2003); osteopontin production which was associated with increased resorption pit formation (Terai et al. 1999); increased IGF-1 mRNA expression associated with increased osteogenic effects (Lean et al. 1995) and downregulation of glutamate and aspartate transporter (GLAST) mRNA expression, pointing to a paracrine role of

glutamate in the communication between osteocytes and osteoblasts (Mason et al. 1997). Although the exact mechanism by which the mechanical force is recognized by cells in bone and transduced into a biochemical signal is unknown, membrane spanning proteins that anchor the cells to its extracellular environment such as integrins have been suggested to play a role in the mechanotransduction process. Mechanical stimulation affects the integrins, which act as mechanoreceptors, activating signalling pathways and affecting gene expression (Rubin J et al. 2006).

Several hormones that are known to regulate the bone turnover process have been demonstrated to also affect osteocyte survival and function following binding to their receptors expressed on the osteocyte (**Table 1.2**). Osteocytes have been shown to undergo apoptosis in response to glucocorticoids both in vivo (Weinstein et al. 1998) and in vitro (Kogianni et al. 2004); respond to PTHrP and PTH binding (Rao et al. 1983, Fermor et al. 1995), which were shown to alter cx43 expression and induce osteocyte apoptosis possibly regulating osteocyte communication and survival (Divieti et al. 2001). Osteocytes were also shown to respond to intermittent PTH administration by downregulating expression of sclerostin, which is normally thought to maintain bone lining cells in a quiescent state on the bone surface, therefore allowing the lining cells to return to an active bone forming state (Keller et al. 2005, Poole et al. 2005). In addition, osteocytes have been shown to express receptors for 1, $25(OH)_2D_3$ (Boivin et al. 1987) and the androgens testosterone and DHEA (Notelovitz et al. 2002).

Furthermore, osteocytes have been shown to undergo apoptosis in response to estrogen loss induced by either gonadotrophin-releasing hormone analogs or ovariectomy (Tomkinson et al. 1997) which could be reversed following the administration of estrogen (Tomkinson et al. 1998). The effect of estrogen on osteocytes is reviewed in §1.11.8 and is the subject of study in chapters 3, 4 and 6.

Species	Number of osteocytes/ mm^2	References	Number of osteocytes /mm^3	References
TRABECULAR BONE				
rat	942.8 ± 49.5	Mullender et al. 1996 Tomkinson et al. 1998	93,200 ± 5400	Mullender et al. 1996
rabbit	679.2 ± 68.5	Mullender et al. 1996	61,500 ± 6800	Mullender et al. 1996
monkey	400.1 ± 47.9	Mullender et al. 1996	37,300 ± 4800	Mullender et al. 1996
pig	399.5 ± 65.4	Mullender et al. 1996	43100 ± 7600	Mullender et al. 1996
cow	294.8 ± 24.4	Mullender et al. 1996	31900 ± 2900	Mullender et al. 1996
human	271.3 ± 28.2 190 ± 22	Mullender et al. 2005 Qiu et al. 2003	13,100 ± 2400	Mullender et al. 1995
CORTICAL BONE				
human osteoporotic human	~ 600 ~150	Vashishth et al. 2002 Tomkinson et al. 1997	Unknown	Unknown
dogs	~ 600	Frank et al. 2002	Unknown	Unknown
horse	510 ± 53 – 640 ± 71	Skedros et al. 2005	Unknown	Unknown
elk	643 ± 50 – 732± 55	Skedros et al. 2005	Unknown	Unknown
sheep	546 ± 54- 710 ± 65	Skedros et al. 2005	Unknown	Unknown
mouse	8wks: 402.5 ±47.1 16wks: 587.4±41.6	Garris et al. 2006	90000 ± 10000	Erlebacher et al. 1998

Table 1.1. Osteocyte density in different species.

Osteocyte Receptors	Function	References
PTH receptor Femor and Skerry 1995 Van der Plas 1994	• Mechanotransduction • Mediates anti-apoptotic effects of PTH • Downregulates sclerostin expression	Divieti et al. 2001 Bringhurst et al. 2002 Bellido et al. 2005
Glucocorticoid receptor (GR) Abu 1997, Gu 2005	• Mediates apoptosis in vivo and in vitro	Weinstein et al. 1998 Kogianni et al. 2004
Estrogen receptors (ER) **ER alpha** Braidman 1995,Hoyland 1997 Vidal 1999 **ER beta (§1.11.4)**	• Mechanotransduction (§1.11.4) • Uncertain function	Ehrilch et al. 2002 Riggs et al. 2002
FAS receptor In vivo (Hatakeyama 2000) In vitro (Kogianni 2004)	• Mediates apoptosis in vitro (MLOY-4)	Kogianni et al. 2004
CD 40 receptor	• Regulates production of matrix metalloproteases	Ahuja et al. 2003
Neurokinin-1 receptor	• Possibly regulates osteoclastic resorption	Goto et al. 1998
Glutamate receptor	• Mechanotransduction	Mason et al. 1997
Serotonin receptor (5-HT_{2B})	• Mechanotransduction	Westbroek et al. 2001
Vitamin D receptor (VDR) Boivin 1987,	• Anti-apoptotic effects • Increase osteocalcin production in MLO-Y4 osteocytes	Lohmann et al. 2000
Androgen receptors		Abu et al.1997, Notelovitz et al.2002 Braidman et al. 2000
Prostaglandin receptors	• Mechanotransduction	Cherian et al. 2003

Table 1.2. Receptors identified in osteocytes and their proposed functions.

1.8 Cell death

Cell death can be distinguished into necrotic cell death, which refers to the morphology seen when cells or tissues die from severe physical, chemical or toxic damage accompanied by inflammation and changes in tissue architecture and apoptotic cell death, a form of programmed cell death, which is the result of the induction of an internal suicide program.

Apoptosis is a physiological and essential homeostatic mechanism for the healthy development and maintenance of tissues (Kerr et al. 1972, Levine et al.1994, Cotman and Anderson 1995) by regulating the size and the functions of organs through the removal of harmful, excess or damaged cells (Hall et al. 1994, Pradhan et al. 1997). Apoptosis, as defined in literature, is characterised by membrane blebbing, chromatin condensation, DNA cleavage and fragmentation of the cell into apoptotic bodies. The contents of dying cells are eliminated by phagocytosis in order to avoid the generation of an inflammatory response within the body (Kerr et al.1972, Savill et al. 1997). Apoptosis usually involves an initiating phase, decision, execution and clearing phase (Guchelaar et al. 1997) although the exact sequence of events in the apoptotic cycle is now thought to be less clearly defined and to involve some complex interactions (decision events) between the cell destined to be apoptotic and nearby phagocytes (Reddien et al. 2001). Apoptosis is stimulated by a number of stimuli including lack of extracellular survival factors, administration of steroid hormones, viral infections, oxidative stress, ionising radiation and DNA damage and is regulated by the function of several proteins such as the bcl-2 proteins, death receptors, the p53 protein and the caspases (Dragovich et al. 1998).

Caspases (Cysteine ASpartate ProteASES), are cysteine proteases that cleave their target molecules at very specific sites, following aspartate residues (asp-x). (Allen, et al. 1998, Thornberry et al. 1998). Caspases play a central role in the apoptotic machinery as they mediate the apoptotic process in cells from the initial stages (initiator caspases) to their package in apoptotic bodies for elimination by phagocytosis (effector caspases). Initiator

caspases (caspases 1, 2, 8, 9 and 10) are activated mainly by death receptors and by functional changes occurring in the mitochondria (Dragovich et al. 1998, Chen et al. 2002). For example, caspase 8 is recruited during initial phases of the apoptotic cascade following the binding of FAS ligand to FAS death receptor enabling the activation of effector caspases further down the apoptotic cascade. The latter include caspases 3, 6 and 7 which mediate the execution phase of apoptosis (Dragovich et al. 1998, Allen et al. 1998, Decaudin et al. 1998). For example, activated caspase-3 causes proteolytic cleavage of the DFF45 enzyme (DNA fragmentation factor 45) in humans, or ICAD (inhibitor of caspase activated Dnase) in mice, which are inhibitors of DFF40 and CAD respectively, allowing CAD and DFF40 to enter the nucleus and degrade DNA into oligomers of 180bp (Guchellar et al. 1997) by activating endogenous Ca^{++}/Mg^{+} dependent endonucleases that cleave DNA into nucleosomes) (Guchelaar et al. 1997). In addition, caspases carry out the cleavage of proteins such as PAK-2 and gelsolin and actin filaments and result in the disassembly of the cytoskeleton (Thornberry et al. 1998, Allen et al. 1998). Caspases are also thought to be responsible for cleavage of proteins involved in the repair and replication of DNA, such as PARP and replication factor C, resulting in DNA damage and degradation by endonucleases (Thornberry et al. 1998, Allen et al. 1998). In general, caspases disassemble the cell's structure, terminate DNA replication, attack the nucleus, prevent repair mechanisms and prepare the cell for phagocytosis.

1.9 Apoptosis and bone

In bone there is evidence of both necrotic and apoptotic cell death. For example, necrosis has been observed in response to exposure to radiation (Sugimoto et al. 1993), steroid use (Watanabe et al. 1989), ischemia (Rosingh and James 1968) and leads to the formation of dead bone patches separated from healthy bone (Wong et al. 1987). Furthermore, there is evidence indicating that chondrocytes and bone cells such as osteoclasts, osteoblasts and osteocytes undergo apoptosis during development, bone remodelling as well as in disease situations indicating that apoptotic cell death is important for the skeletal development and maintenance throughout life, as described in

the following paragraphs. Hypertrophic chondrocytes present during endochondral ossification and fracture healing undergo apoptosis during the replacement of cartilaginous tissue by woven bone (Lee et al. 1998) possibly in order to signal to osteoclasts for their removal from the area. However, the mechanism is not clear yet (Bronckers et al. 2000). Osteoclasts have been shown to undergo apoptosis following the completion of the resorption process (Hughes and Boyce 1997, Xing and Boyce 2005) as well as in response to anti-resorptive agents such as estrogen and bisphosphonates (Kameda et al. 1997, Hughes et al. 1996, 1995; Papapoulos et al. 1997, Rodan 1998, Rogers et al. 1999).

Furthermore, osteoblasts have also been observed to undergo apoptosis at the end of bone formation in order to regulate the number of osteoblasts present during bone remodelling (Jilka et al. 1998, Landry et al. 1997) or in response to glucocorticoids in mouse (Weinstein et al., 1998, Plotkin et al., 1999) and human (Weinstein et al. 1998), but not in rat (Silvestrini 2000) and to various cytokines in vitro such as TNF-a, IL-1, IFN-γ (Ozeki et al. 2002) and FasL (Kawakami et al. 1997, Jilka et al. 1998).

1.10 Apoptosis and osteocytes

It has been demonstrated that apoptotic death of osteocytes, characterised by DNA fragmentation, chromatin condensation and cell shrinkage in both pathological and healthy bone (Noble et al. 1997), is associated with regions of damaged bones or regions characterised by increased bone modelling and remodelling activity. For example, increased incidence of apoptotic osteocytes has been observed in growing bone characterised by increased bone resorption (Noble et al. 1997) as well as in response to estrogen deficiency induced by either gonadotrophin-releasing hormone analogues (GnRHa) or ovariectomy (Tomkinson et al. 1997, 1998) indicating that the increase in bone turnover following estrogen loss is associated with high prevalence of apoptotic osteocytes. These indices of apoptosis were reversed following estrogen administration in both humans and rats (Tomkinson et al. 1997, 1998). More recent studies have also shown that estrogen deficiency induces osteocyte apoptosis in ovariectomised mice (Kousteni et al. 2001).

Intermittent administration of PTH has been suggested to reduce osteocyte apoptosis in mouse vertebra in vivo and in MLO-Y4 osteocytes in vitro (Jilka et al. 1999). However, other studies have suggested that PTH induces a transient increase in osteocyte apoptosis in the distal femur (Stanislaus et al. 2000) indicating that further work is required in order to identify the exact role of PTH in osteocyte apoptosis.

In addition, in vivo evidence of increased osteocyte apoptosis has been demonstrated in response to prolonged use of glucocorticoid treatment in humans and mice and could be associated with the pathogenesis of osteonecrosis (Weinstein et al. 2000) and osteoporosis (Weinstein et al. 1998). In vitro, apoptosis of osteocytes has been observed in response to dexamethasone (Plotkin et al. 1999, Kogianni et al. 2004, Kousteni et al. 2001), etoposide and TNF-α treatment (Plotkin et al. 1999; Kousteni et al. 2001) and to be reversed following treatment with bisphosphonates (Plotkin et al. 1999, Kogianni et al. 2004) or estrogen (Kousteni et al. 2001, Gu et al. 2005). In addition, Kogianni et al has suggested that the dexamethasone-induced apoptosis was characterised by upregulated Fas and ERK protein expression as well as the involvement of caspases 8 and 3/7 in osteocytes (Kogianni et al. 2004). Furthermore, it has been suggested that hydrogen peroxide induces apoptosis with the involvement of caspase 3 activation in osteocytes (Kikuyama et al. 2002).

Large numbers of apoptotic osteocytes have also been identified surrounding resorption areas induced by fatigue microdamage (Verborgt et al. 2000) or during orthodontic tooth movement (Hamaya et al., 2002). Furthermore, unloading has also been linked to an increase in osteocyte apoptosis in murine (Aguirre et al. 2006) and rat bone (Basso et al. 2006) in vivo and in vitro (Bakker et al. 2004), shown in rat bone to be reversed upon reloading (Basso et al. 2006). Furthermore, Noble et al have shown that there is a U-shape relationship between osteocyte survival and the strains they experience, with normal physiological strains reducing apoptosis, whereas damagingly high levels of loading induced apoptosis. In the latter case, the transient increase in the proportion of apoptotic osteocytes in response to high levels of mechanical loading was immediately

normal physiological strains reducing apoptosis, whereas damagingly high levels of loading induced apoptosis. In the latter case, the transient increase in the proportion of apoptotic osteocytes in response to high levels of mechanical loading was immediately followed by subsequent intracortical remodelling providing the first evidence that the presence of apoptotic osteocytes within bone might regulate specific site-directed remodelling (Noble et al. 2003). In addition, studies have demonstrated that bones containing low osteocyte numbers such as the ear ossicle (Marotti et al. 1998) and the labyrinthe capsule (Kakizaki et al. 1971) have been characterised by reduced remodelling (Marotti et al. 1998) and increased accumulation of microdamage (Mori et al. 1997). These findings indicate that osteocyte apoptosis is a process observed both in normal and pathological bone conditions and it might be essential in influencing the remodelling process towards specific sites.

1.11 Estrogen

1.11.1 General background

Estrogens are a group of steroid hormones that include the 17β-estradiol (E_2) and the estrone (E_1) mainly produced by the ovaries during puberty and by the adipose tissue during menopause respectively, and estriol (E_3) produced by the fetoplacental unit during pregnancy (Dotsch et al. 2001).

The production of 17β-estradiol, the main endogenous estrogen in the human body, varies throughout the phases of the menstrual cycle, so that it is produced at low levels during the early follicular phase (110-1100 pmol/l), reaches a peak at the mid-cycle (1100-1450 pmol/l) and decreases to intermediate levels (740pmol/l) in the luteal phase (Dotsch et al. 2001). At menopause, the serum concentration of 17β-estradiol falls dramatically to levels below the early follicular phase levels (commonly below 110 pmol/l). During this time, the major estrogen in the body is estrone (E_1) produced by the aromatisation of androgen precursors in the adipose tissue (Dotsch et al. 2001).

Estrogen (E_2) exerts multiple effects on a variety of tissues in the body. For example, it has been shown to influence the growth, differentiation and function of the reproductive system and the female mammary gland (Dotsch et al. 2001), promote sexual differentiation in the mammalian brain and modulate mood, behaviour (Genazzani et al. 1998) and memory performance (Luine et al. 1985). In addition, estrogen has been shown to prevent oxidation of low density lipoproteins (LDL) in the cardiovascular system (Miller et al. 1996, Perrela et al. 2003), to induce relaxation of the vascular smooth muscle, and to promote and maintain body fat distribution, an effect lost during postmenopause (Ley et al. 1992). The effects of estrogen on the skeletal system are reviewed in more detail in §1.11.4.

1.11.2 Estrogen Receptors (ERs) and bone

Most of the skeletal effects of estrogen are known to be mediated following binding to estrogen receptors (Couse and Korach 1999). In bone, both ERα and ERβ have been localised on osteoclasts, osteoblasts and osteocytes (Braidman et al. 1995; Hoyland et al. 1997, Vidal et al. 1999, Westbroek et al. 2000) but also on chondrocytes possibly mediating the effects of estrogen on the fusion of the epiphyseal growth plate (Sims et al. 2002, Bord et al. 2001). However, differences have been reported in the expression levels of the two ER subtypes in these cells. For example, in primary neonatal rat osteoblasts, the level of ERβ mRNA was found to be higher than the ERα mRNA levels which in turn were gradually increased following the differentiation of osteoblasts to mature osteoblasts (Onoe et al. 1997). These findings suggested that the levels of ER expression could change during the differentiation process of the same cells. In addition, it has been observed that cells at different sites within the same tissue might respond differently to estrogen due to alternative expression of distinct patterns of ER subtypes; For example, ERβ was found to be predominantly expressed in osteoblasts and to a minor extend in osteoclasts and osteocytes in cancellous bone, whereas ERα was predominantly expressed in osteoblasts and osteocytes in cortical bone (Bord et al. 2001) possibly suggesting that the two types of ERs might mediate different functions.

whereas, ERβ knockout male mice (βERKO) exerted no phenotypical differences (changes in BMD) compared to the wild type mice (Windhal et al. 1999, Vidal et al. 2000). In contrast to male ERβ KO mice, adult female ERβ KO mice showed a slightly elevated BMD (Windhahl et al. 1999) and an increase in cortical bone (Widhal et al. 2001), while aged βERKO mice were also protected against the age-related bone loss observed in the wild type mice (Ke et al. 2002). These studies indicated a possible inhibitory role of ERβ in bone growth and bone formation during adolescence and the prevention of bone loss associated with ageing in female mice.

There are various experimental models for investigating postmenopausal bone loss, such as the rat, mouse, dog and primates. The use of dogs is controversial and primates are not practical to most research groups. The most frequently practiced in vivo model is the rat model of OVX (Frost and Jee 1992), since it is characterised by changes that mimic the response of the human skeleton to estrogen deficiency. Most studies have demonstrated that, in a similar way to humans, estrogen loss in mature rats results to bone loss due to abnormal turnover (Wronski et al. 1988). In contrast to the rat model, the responses of the mouse and human skeleton to estrogen are different. For example, estrogen loss does not affect bone growth in mice possibly indicating that the murine periosteal osteoblasts are less responsive to estrogen than in humans (Turner 1999).

1.11.3 Estrogen receptors and osteocytes

Even though both ERs have been identified in osteocytes (Braidman et al. 1995, Hoyland et al. 1997, Vidal et al. 1999), it has been demonstrated that both primary (Westbroek et al. 2000) and MLO-Y4 osteocytes (Matikainen and Vaananen 1999) preferentially express ERα. Expression of ERa has been shown to be reduced following estrogen withdrawal (Hoyland et al. 1999) and idiopathic osteoporosis in humans (Braidman et al. 2000) and ovariectomy in rats (Lim et al. 1999) possibly indicating that the concentration of estrogen in the serum might be regulating the expression of ERα. In addition, in vivo studies have also shown that the expression of ERα in osteocytes is enhanced in response to mechanical loading (Ehrlich et al. 2002) indicating that this receptor might be involved in the osteocytic response to mechanical strain.

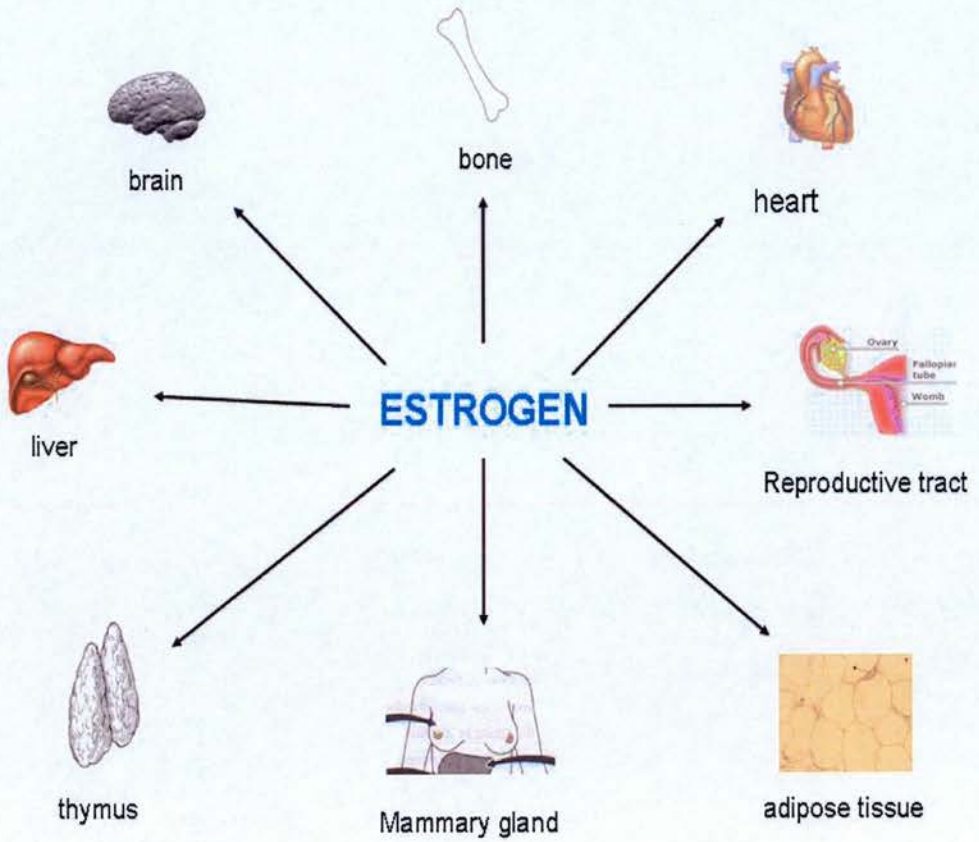


Figure 1.4. Sites of estrogen action. Estrogen affects the growth and function of a number of organs in the body including the heart, brain, liver, thymus and bone (for review see Dotsch et al. 2001).

1.11.4 Effects of estrogen on bone tissue

Estrogen has been shown to exert multiple effects on the skeletal system such as modulating the size and shape of the skeleton and maintaining skeletal microarchitecture during the adult life (Turner et al. 1994; Couse and Korach, 1999).

Bone mass increases throughout childhood and adolescence (Compston et al. 2001) and reaches a peak in the third decade of life, indicating that the accrual of bone mass during puberty and growth is tightly regulated by the presence of estrogen. Peak bone mass, which is also affected by diet, exercise and genetic background (Ralston et al. 1997), is maintained until the fifth decade of life when age-related as well as menopausal bone loss commence. Even though bone loss continues throughout life for both sexes (0.3%-0.5% of their bone mass every year), the rate of bone loss is accelerated in females at the time of the menopause and decreases after 5 to 10 years (Schneider et al. 1997).

1.11.5 Estrogen deficiency and bone

Estrogen deficiency that occurs during late menarche, amenorrhea caused by anorexia nervosa and excessive exercise, disease or drug treatment, and natural or induced menopause has been shown to be harmful to the attainment of peak bone mass during adulthood and to result in osteoporosis characterised by low bone mass and increased fracture risk (Aitken et al. 1973, Riggs et al. 2002, Cummings et al.1998). These findings indicate that the development of osteoporosis depends both on the estrogen-regulated peak bone mass attained during growth and the subsequent rate of bone loss (Riggs 1991).

At the tissue level, estrogen deficiency has been associated with changes in the equilibrium between formation and resorption leading to increased bone resorption and turnover activity and impaired bone formation within each remodelling cycle (Wronski et al. 1988; 1989, Raisz 2005). The consequences of E_2 deficiency involve a biphasic pattern of bone loss characterised by an initial transient increase in bone loss followed

by a gradual chronic phase of bone loss in postmenopausal women (Riggs et al. 1991) and in ovariectomised rats (Wronski et al. 1989, Kalu et al. 1991).

Recent findings also suggest that estrogen deficiency could upregulate the immune system by activating and expanding TNF-a-producing T cells, which is a key step in estrogen deficiency-driven bone loss regulated by transforming growth factor-β (TGF-β), interleukin-7, and interferon-γ (Weitzmann and Pacifici 2005). In addition, there is increasing evidence suggesting that estrogen deficiency could be controlling the generation of reactive oxygen species (ROS) since upregulated production of ROS during estrogen deficiency is implicated in estrogen deficiency-induced bone loss, further analysed in Chapter 2.

1.11.6 Hormone Replacement Therapy (HRT)

HRT is generally used for the management of osteoporosis in women with acute menopausal symptoms (hot flushes, night sweats) and in postmenopausal women who prefer HRT over other treatments such as bisphosphonates, raloxifene, strontium ranelate.

Long-term hormone replacement therapy has been shown to prevent postmenopausal bone loss (Lindsay et al. 1976, Christiansen et al. 1980, Grady et al. 1992) by reducing bone turnover (Compston J et al. 2001, Grady et al. 1992), the activation frequency and the size of the resorption cavities in postmenopausal women (Vedi and Compston 1996). Although long-term estrogen administration (26-42 months) resulted in reduced bone resorption followed by reduced bone formation, short-term studies of HRT (2.5-4 months) to postmenopausal women demonstrated a significant reduction only in bone resorption and not in bone formation observed. These findings indicate that, in contrast to bone resorption which is rapid and could be observed only in shorter period of time, bone formation requires a longer period in order to be completed (Riggs et al. 1972). In a similar way to postmenopausal women, estrogen administration to ovariectomised

(OVX) rats not only managed to provide complete protection against bone loss, but to significantly reduce all of indices of bone turnover as well (Wronski et al. 1988).

HRT has also been shown to reduce clinical vertebral (Lindsay et al. 1980, Lufkin et al. 1992) and non-vertebral fractures (Weiss et al. 1980, Michaelsson et al. 1998) in HRT users. Evidence from the WHI study has shown that HRT is important for fracture protection in healthy postmenopausal women (Writing Group of the Women's Health Initiative Investigators 2002, The Women's Health Initiatiave Steering Committee 2004) within the first decade following menopause when administered alone or combined with progestogens (Naessen et al. 1990).

1.11.7 Estrogen and bone cells

The presence of estrogen prevents bone loss by reducing osteoclastogenesis or by inducing osteoclast apoptosis either by downregulating the production of the osteoclastogenic factors IL-1, IL-6 (Girasole et al. 1992, Passeri et al. 1993, Manolagas et al. 1995, 2000), RANKL (Hofbauer et al. 1999), MCSF (Srivastava et al. 1998) and TNF-α (Srivastava et al. 1999) and upregulating TGF-β production by the stromal/osteoblastic cells (Hughes et al. 1996) or by directly binding to ERs (Kameda et al. 1997, Sunyer et al. 1999). In addition, the inhibitory effect of estrogen on osteoclastogenesis could be associated with the upregulation of osteoprotegerin (OPG) production by the stomal/osteoblastic cells that blocks the binding of the RANKL to RANK on osteoclasts (Bord et al. 2003, Michael et al. 2005).

Estrogen has also been shown to upregulate the expression of collagen type I (Ernst et al. 1988), alkaline phosphatase (ALP) (Chen et al. 2002), OPG, IGFI (Ernst et al. 1989, Gray et al. 1989) and TGF-β gene in osteoblasts (Tau et al. 1998). Even though there is no evidence that estrogen administration at bone sparing doses increases bone formation, studies have suggested that administration of high estrogen doses have anabolic skeletal effects in postmenopausal women (Vedi et al, 1999, Tobias and Compston 1999) as well

effects in postmenopausal women (Vedi et al, 1999, Tobias and Compston 1999) as well as in animal models (Edwards et al. 1992) by increasing the proliferation, differentiation and function of osteoblasts (Manolagas 2000). In vitro studies have also shown that estrogen induces the proliferation of rat (Ernst et al. 1988, Cheng et al. 2002) and human osteoblasts (Ikegami et al. 1994, Chen et al. 2002). Furthermore, both in vitro and in vivo studies have shown that estrogen prevents glucocorticoid-induced apoptosis of osteoblasts following the end of the formation period (Kousteni et al. 2001) possibly through the increased production of TGF-β (Karsdal et al. 2002).

1.11.8 Estrogen and osteocytes

Estrogen deficiency due to gonadotrophin-releasing hormone analogs (GnRH) in postmenopausal women or ovariectomy in rats has been shown to induce an increase in osteocyte apoptosis (Tomkinson et al. 1997, 1998) which was reversed following estrogen administration (Tomkinson et al. 1998). These studies along with other in vivo studies on OVX mice (Kousteni et al. 2001) supporting the negative effects of estrogen deficiency on osteocyte apoptosis, have indicated that the presence of estrogen is important in the control of osteocyte apoptosis as well as in the maintenance of osteocyte viability.

In addition, estrogen has been shown to protect osteocytes against glucocorticoid-induced apoptosis (Kousteni et al. 2001, Gu et al. 2005), etoposide and TNF-α induced apoptosis in vitro (Kousteni et al. 2001) indicating that estrogen is associated with pro-survival effects against a number of death-inducing stimuli in osteocytes. In vitro studies have demonstrated that the release of TGF-β by the osteocytes was enhanced in response to estrogen in order to suppress osteoclastic bone resorption (Heino et al. 2002) possibly indicating that osteocytes could be mediating the inhibitory effect of estrogen on bone resorption.

1.12 Selective Estrogen Receptor Modulators (SERMs)

1.12.1 General background

The use of HRT exerts beneficial effects on bone as discussed in §1.11.6. However, the chronic use of estrogen as a part of HRT has also been associated with an increased risk of endometrial (Grady et al. 1995) and breast cancer (Colditz et al. 1995). Furthermore, agents that exert bone-specific effects like calcitonin or bisphosphonates have been widely used to prevent postmenopausal bone loss in established osteoporosis (Gennari et al. 1998, Whiteman et al. 1999) without however exerting any positive extraskeletal effects on postmenopausal women. These findings point to the need of the design of new molecules that would demonstrate tissue selective activities (McDonnell et al. 2000, Sandberg et al. 2002) and be efficiently used for the treatment of postmenopausal osteoporosis. During the last decades, Selective Estrogen Receptor Modulators (SERMs) have played an important role in the current therapeutic approach against menopause-associated conditions.

SERMs are synthetic non-steroidal compounds that produce estrogen-like effects and can act as estrogen agonists in some target tissues such as bone, nervous and cardiovascular system, while they may also exhibit estrogen antagonism and/or minimal agonism in reproductive tissues such as the breast or uterus (Kauffman et al. 1995, Dodge et al. 1997, McDonnell et al. 2000, Sandberg et al. 2002). SERMs are categorised according to their structure into the triphenylethylenes such as clomiphene, tamoxifen, droloxifene and idoxifene, the benzothiophenes such as raloxifene and the LY 117018 SERM (analysed in more detail in Chapters 3 and 5), the benzopyrans such as levormeloxifene and the dehydronapthalenes such as nadoxifene (Agnusdei and Iori 2000).

MER-25, an anticholesterol agent, was the first SERM introduced into the market in the 1950s (Lerner and Jordan 1990) and was shown to inhibit the stimulatory effects of estrogen without itself inducing any stimulatory effects on the breast tissue. However, MER-25 was soon removed from the market due to increased toxicity and low potency

(Lerner 1981) while derivatives of MER-25, such as clomiphene citrate and tamoxifen, were introduced clinically in the 1970s. Tamoxifen has been extensively used for the treatment of breast cancer in postmenopausal women (Cole et al. 1971) but its long-term use has been associated with increased risk of endometrial cancer (Friedl et al. 1994) and uterine stimulation (Fisher et al. 1994) indicating that the tissue selective effects exerted by tamoxifen are not free of adverse effects on other reproductive tissues.

Interestingly, SERMs such as raloxifene were demonstrated to be effective against advanced breast cancer (Hol et al. 1997) and to block the uterotrophic effect of estrogen (Black et al. 1993, 1994) or tamoxifen (Jordan and Gosden 1983, Black and Goode, 1980, 1981). These findings suggest that the use of SERMs, such as raloxifene, could be ideal in order to study their beneficial effects on non-reproductive tissues such as bone, as reviewed in §1.13.2.

1.12.2 SERMs and bone

The beneficial effects of raloxifene on bone have been extensively described in estrogen-deficient rats (Sato et al. 1994, 1995, 1996; Evans et al. 1994, 1996) and were demonstrated to be comparable to the effects induced by estrogens such as 17α ethinyl estradiol (Evans et al. 1994, 1996; Sato et al. 1995, 1996a, 1996b) or 17β-estradiol (Sato et al. 1994) used in the same studies. For example, raloxifene treatment was shown to prevent the OVX-induced loss of BMD in the femur and tibia (Black et al.1994) and to increase bone strength and BMD in the lumbar vertebrae (Turner et al. 1994) to a similar extend to estrogen administration without however exerting any negative effects on the uterus (Black et al. 1994, Turner et al. 1994). Comparative studies between the effects of estrogen, raloxifene, tamoxifene and the bisphosphonate alendronate demonstrated that all these antiresorptive agents effectively inhibited bone loss to the same extent by reducing bone resorption and bone turnover. However, raloxifene, in contrast to estrogen, had no effect on the uterus while it also appeared to lower the cholesterol levels in the serum (Frolik et al. 1996, Sato et al. 1996b). These studies on OVX rat models have provided a strong parallel between the anti-osteopenic effects of raloxifene

and estrogen and have indicated that raloxifene acts as an estrogen agonist in bone without negatively affecting the reproductive system (Black LJ et al. 1994, Kauffman et al. 1995, Frolik et al. 1996, Draper et al. 1996).

Treatment of postmenopausal women, with or without established osteoporosis, with raloxifene has been shown to decrease biochemical markers of turnover such as alkaline phosphatase and serum osteocalcin (Draper et al. 1996, Lufkin et al. 1998), and to increase the BMD in the lumbar spine and hip (Delmas et al. 1997, Lufkin et al.1998). In addition, in early postmenopausal women treatment with raloxifene reduced bone resorption to levels comparative to estrogen treatment (Heaney et al. 1997) indicating that raloxifene preserves bone mass possibly by affecting mechanisms similar to those operative in postmenopausal women receiving HRT (Weinstein et al. 2003). Furthermore, raloxifene treatment has been shown to also exert positive effects in late postmenopausal osteoporotic women up to 80 years of age by reducing the risk of fracture and increasing the BMD in the spine and femoral neck (Ettinger et al. 1999).

LY 117018, an analogue of raloxifene, has also been shown to act as an antiestrogen in order to prevent estrogenic activity on the uterus of ovariectomised (OVX) rodents (Black and Goode 1981, Jordan and Gosden 1983) and to completely prevent high turnover osteopenia caused by oophorectomy (Bowman et al. 1996). The effects of LY 117018 on bone are the subject of study in Chapter 3 in this thesis.

Other SERMs have also been shown to prevent bone loss and reduce serum cholesterol levels, while the SERM CP336156 has been shown to increase BMD in combination with the anabolic agents CP424391 and PTH in ovariectomised rats (Ke et al. 1998). Tamoxifen has also been shown to preserve bone mass in ovariectomised rats (Turner et al. 1987, Kalu et al. 1991, Grey et al. 1995) and to reduce bone resorption (Steward and Stern 1986; Jordan et al. 1987) and to stimulate cancellous bone formation in intact female mice (Perry et al. 2005). Tamoxifen administration in healthy postmenopausal women reduced serum cholesterol and increased bone mineral density (Chang et al.

1.13 Summary

Bone undergoes continuous remodelling cycles in order to sense structural and mechanical requirements, adapt its structure to suit its function and to repair damage (Wolf 1892, Turner 1992, Parfitt 1994). Osteocytes are thought to play an important role in the bone remodelling process since high osteocyte density has been associated with regions characterised by resorption and formation activity (Power et al. 2002) while absence of osteocytes is associated with low occurrence or absence of remodelling surfaces (Marotti et al. 1998). In addition, there is evidence accumulating that the apoptotic death of osteocytes might be providing the signals that direct turnover since high incidence of osteocyte apoptosis has been associated with increased resorptive activity in response to unloading or excessive loading (Verborgt et al. 2002; Noble et al. 2003), glucocorticoid treatment (Weinstein et al. 1998, 2000) or estrogen deficiency (Tomkinson et al. 1997, 1998). Estrogen deficiency has been shown to lead to osteoporosis characterised by low bone mass and increased fracture risk (Aitken et al. 1973, Riggs et al. 2002, Cummings et al.1998). These observations suggest that it might be beneficial to prevent excess osteocyte apoptosis that might lead to pathological conditions.

This thesis investigates compounds, such as the SERMs, that might lead to osteocyte survival in a similar manner to the known protective effects of 17β-estradiol. In addition, the possible mechanism of action of these compounds, as antioxidants, on osteocytes is discussed in Chapter 2 and is investigated in Chapters 4 and 5.

CHAPTER 2

Antioxidant molecules and bone

2.1 Mechanism of estrogen action

As described in the previous chapter, estrogen (E_2) has been shown to elicit diverse effects on a variety of tissues in the body, which are believed to be mediated through genomic or non-genomic mechanisms depending on whether gene transcription takes place following the interaction of the ligand with intracellular, nuclear estrogen receptors (ERs) **(Figure 2.1).**

The most well characterised genomic mechanism of estrogen action, also known as the classical mode of estrogen action, involves the diffusion of estrogen across the plasma membrane and its interaction with intracellular nuclear ERs (**Figure 2.1**) of the subtypes α and β present in an inactive form either in the cytosol or the nucleus. Following binding of E_2, the receptor complex undergoes dimerization and conformational changes in order to act as a transcription factor and bind to cognate Estrogen Response Elements (EREs) in the promoter region of the target genes enhancing gene transcription (Tsai et al. 1998, Razandi et al. 1999). However, not all estrogen receptors are involved in the classical mechanism of estrogen action since three of the five recently identified ERβ isoforms (ERβ 3-5) have not been demonstrated to interact with estrogen or to drive ERE-dependent gene transcription (Moore et al. 1998). Paradoxically, ERE-dependent gene transcription could also occur following the activation of the estrogen receptor by growth factor signalling (**Figure 2.1**). For example, it has been demonstrated that EGF could activate the estrogen receptor through the MAP-Kinase pathway (Bunone et al. 1996) indicating that the ER could demonstrate activity independently of estrogen binding (Hall et al. 2001).

In addition, the estrogen-ER complex could also induce the expression of genes lacking EREs in their promoter region by indirect binding of the complex to cognate DNA binding sites by interacting with transcription factors such as the fos/jun transcription complex on AP-1 sites, NF-kB and Sp-1 (ERE-independent pathway) (Webb et al. 1995, Saville et al. 2000). These ERE- and ligand-independent mechanisms of estrogen action along with the non-genomic pathway (Pietras & Szego 1975; 1977, Wehling et al. 1997,

Collins & Webb 1999, Levin et al. 1999, Falkenstein et al. 2000) constitute the non-classical mode of estrogen action.

The non-genomic actions of estrogen can be distinguished into the ER-mediated and the non ER-mediated mechanisms (Wehling et al. 1997). In the former mechanism, estrogen is thought to interact with a cell surface form of estrogen receptor (**Figure 2.1**) (Pietras and Szego 1977), the precise nature of which is still not well characterised. Studies have suggested that the membrane ER might be a variant form of ERα that has translocated to the plasma membrane, where it is thought to activate survival pathways such as the MAPK (Kousteni et al. 2001) or PI3-Kinase signalling pathways (Levin et al. 2002). However, further studies are required in order to characterise the exact nature and function of this membrane receptor as well as the significance of E_2 binding to an ER without entering the cytoplasm.

The non ER-mediated mechanisms involve the interaction of E_2 with membrane receptors such as the insulin like growth factor-1 receptor (IGF-1R) and the epidermal growth factor receptor (EGFR) (Kahlert et al. 2000, Filardo et al. 2002) leading to activation of PI3K, Akt and MAP Kinase pathways enhancing gene transcription (Levin 2002), while E_2 is also thought to interact with the G-protein coupled receptor GRP30 (Filardo et al. 2002, Revankar et al. 2005, Manavathi et al. 2006) activating MAP Kinase pathways (Filardo et al. 2002, Manavathi et al. 2006), (**Figure 2.1**). Furthermore, steroids could also interact with lipids on the plasma membrane affecting membrane fluidity and therefore enzyme activity (Whiting et al. 2000); and with proteins on the membrane known as estrogen binding proteins (EBPs) that trigger changes in intracellular signalling stimulating transcription (Deroo and Koorach 2006).

Furthermore, evidence is accumulating on the action of estrogen as an antioxidant molecule (Behl et al. 1995; 1997, Green et al. 1997, Miranda et al. 2000) and the significance of this mechanism of estrogen action to bone will be discussed in detail in the following paragraphs and in Chapters 2, 4 and 5.

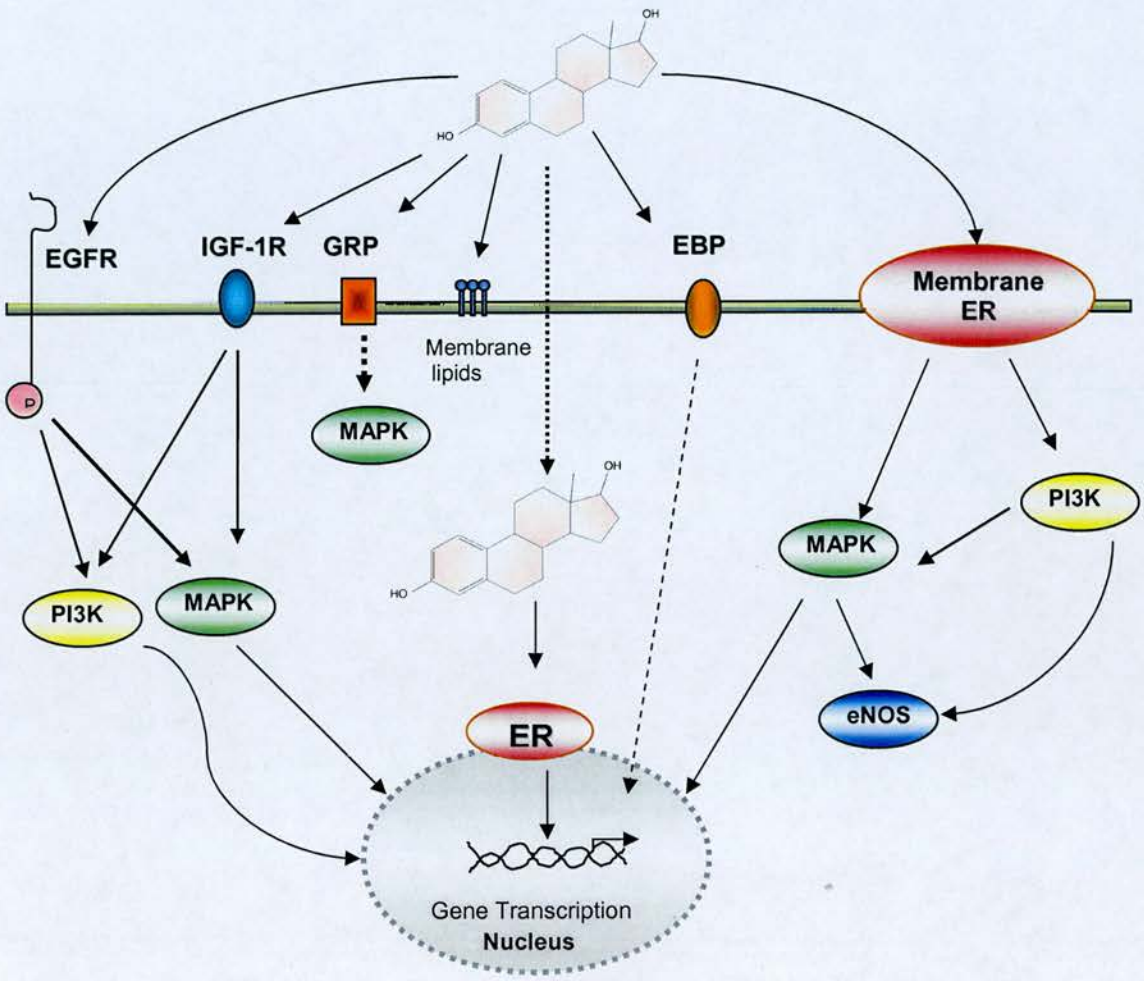


Figure 2.1. Summary of ER-dependent and independent mechanisms of estrogen action.

2.2 Reactive Oxygen Species (ROS)

2.2.1 Free radicals, ROS and oxidative stress

Free radicals are short-lived, highly reactive molecules that contain one or more unpaired electrons in their outer orbit (Halliwell 1991). They interact with other molecules in order to acquire the necessary electron, inducing changes on the chemical structure of these molecules and converting them into free radicals. Free radicals along with molecules that exhibit free radical-like behaviour, such as hydrogen peroxide and the singlet oxygen, are known as oxidants or reactive oxygen species (ROS) and are outlined in **Table 2.1**.

ROS, which sense changes in oxygen concentration, are constantly produced inside the cells under normal metabolic pathways (Harman 1981, Halliwell et al. 1992, Finkel and Holbrook 2000, Balaban et al. 2005), such as during the cellular respiration process that takes place in the mitochondria during which a small percentage of oxygen is converted to the superoxide radical (Richter et al. 1995, Finkel 2000). ROS are also produced in the peroxisomes, which are the site of hydrogen peroxide (H_2O_2) production or also through the action of enzymes with peroxidase activity such as the cytochrome P450 enzymes or enzymes associated with the plasma membrane such as the NADPH oxidase. ROS generation could be enhanced during the process of ageing (Harman et al. 1956), by factors such as radiation (Stief et al. 2003), pollutants, smoking, drugs, chemicals (Halliwell 1997), inflammation (Curnutte et al. 1987), bone fractures (Symons et al. 1996), by the binding of growth factors such as EGF to their receptors (Bae et al. 1997, Cha et al. 2000, Droge et al. 2002) and by the presence of cytokines such as for example TNF-α (Chang-Hoon et al. 2000).

When the generation of endogenous ROS is tightly regulated, ROS exert positive biological roles. For example, they are essential for the defence against anti-microbial insults (Babior et al. 1773, Curnutte et al. 1987), in sensing oxygen tension in the control of ventilation and erythropoietic production (Droge et al. 2002), in regulating functions controlled by O_2 concentration (Chandel et al. 2000), in the maintenance of redox

homeostasis (Droge et al. 2002) and also in signal transduction pathways mediating proliferation, differentiation (Allen and Tresini 2000, Finkel 2003), survival or programmed cell death (Finkel 2003).

Disturbance in the balance between pro-oxidant –antioxidant balance leads to oxidative stress (Sies H. 1991, Behl et al. 2002) which results in cell injury by inducing energy depletion, lipid peroxidation, protein and DNA oxidation and possibly cell death through either apoptosis or necrosis (Finkel 2000). Oxidative stress has been linked to a number of pathological conditions including carcinogenesis (Klaunig and Kamendulis 2004), diseases of the eye such as age-related macular degeneration (AMD) (Beatty et al. 2001) and maturity onset cataract formation (Spector et al. 1984, Taylor and Nowell 1997) and diabetes mellitus (Gorogawa et al. 2002). Oxidative stress is also associated with the pathogenesis of diseases related to inflammation such as rheumatoid arthritis (Jaswal et al. 2003), in atherosclerosis (Parthasarathy et al. 1992, Babior et al. 2000, Finkel 2000, Perrella et al. 2003) as well as in neurodegenerative disorders such as Parkinson's and Alzheimer's disease (AD) (Behl et al. 1992; 1994, Yan et al. 1996).

ROS	Characteristics
Superoxide anion ($O_2^{\cdot-}$)	• Weakly reactive radical • Generates hydroxyl radical ($OH^{\cdot}$) by reaction with H_2O_2 • Produced by activated phagocytic cells to kill bacteria (Curnutte et al. 1987)
Hydroxyl radical ($OH^{\cdot}$)	• Most reactive radical, very short half life (10^{-9} sec) (Jacob 1995) • Produced by superoxide and by H_2O_2 (Fenton reaction) • Stimulates lipid peroxidation by attacking the fatty acids of the phospholipids. Fatty acid chains are then converted to peroxyl radicals, H_2O_2 and lipid hydroperoxides • DNA damage, ATP depletion and gene mutations (Breimer et al. 1991).
Peroxyl radicals (RO_2)	• Produced during lipid degradation
Hydrogen peroxide (H_2O_2)	• More stable ROS than radicals, weak oxidant • Diffuses freely across membranes (Halliwell and Gutteridge 1981) • Generates $OH^{\cdot}$ by reacting with transition metals (Fenton reaction) in cytoplasm and mitochondria (Halliwell and Gutteridge 1992) • Produced by NADPH oxidase in phagocytes and following dismutation of superoxide • Triggers intracellular events (Halliwell and Gutteridge 1981): oxidation of NF-kB (Schreck et al. 1991) and transcription of inflammatory cytokines such as IL-2, IL-6, IL-8 , TNF-α (Baeurele et al. 1988)
Singlet oxygen ($^1O_2$)	• Oxygen with antiparallel spin direction • Highly reactive with lipids; forms peroxide radicals • Limited knowledge on its role

Table 2.1. Important ROS and some of their characteristics.

2.2.2 Oxidants and bone

Both in vivo and in vitro studies have shown that the production of ROS, such as $O_2^{\cdot-}$ and H_2O_2, in response to exogenous stimuli is involved in the formation and activation of murine (Garrett et al. 1990, Suda et al.1993, Fraser J et al.1996, Steinbeck et al. 1998) and rat osteoclasts, respectively (Bax et al. 1992). Under physiological conditions in bone, ROS are produced by osteoclasts through the action of the enzymes NADPH oxidase (Steinbeck et al. 1994) and tartrate resistant acid phosphatase (TRAP) (Vaaraniemi et al. 2004) in order to degrade the calcified tissue (Garett et al. 1990, Sontakke et al. 2002). ROS are also generated during the production of RANKL by the osteoblasts possibly in order to mediate the RANKL-induced cellular events in osteoclasts (Ha et al. 2004).

Some studies have shown that the oxidant status is increased during the inflammatory process associated with bone fractures (Turgut et al. 1999). As the collagen strands break, their ends become exposed to oxygen, which finally form peroxyl radicals affecting and disrupting the cells in the vicinity of the fracture (Symons et al. 1996, Sontakke et al. 2002), while the inflammatory cells recruited to the fracture site contribute to the increase in the oxidant status observed during callus formation (Turgut et al. 1999, Prasada et al. 2003, Yeler et al. 2005) by producing large amounts of superoxide (Finkel 2000). Besides the inflammatory stage of the fracture healing process, ROS generation is also associated with progressive diseases such as rheumatoid arthritis and periodontitis (Madsen et al. 2004) indicating that ROS could be involved in pathological cartilage and bone degradation. In addition, reduced bone mineral density (BMD) observed in pathological conditions such as osteoporosis has also been associated with accumulation of ROS, as evidenced by the use of biochemical markers of oxidative stress (urinary excretion of isoprostanes) (Basu et al. 2001). Furthermore, accumulation of ROS and in particular of hydrogen peroxide observed in ovariectomised mice (Lean et al. 2003) has been proposed to mediate the bone loss associated with estrogen deficiency (Lean et al. 2005). The implication of ROS accumulation in the E_2-deficiency induced bone loss is further discussed in §2.4.2.

Furthermore, ROS have also been observed to reduce bone nodule formation, inhibit the proliferation and differentiation of osteoblasts (Suzuki et al. 1997, Mody et al. 2001, Bai et al. 2004) and bone marrow stromal cells (BMSC) (Bai et al. 2004) and possibly to induce apoptosis in osteoblastic cells in vitro. However, very little is known about the effects of oxidative stress in osteocytes, a subject that will be analysed in Chapter 4 and 5.

2.3 Antioxidant defence system

All organisms have developed defence mechanisms against oxidative stress in order to maintain a balance between the production and clearance rate of free radicals and therefore to maintain cellular integrity (Noguchi et al. 2000, Floyd et al. 1999). These mechanisms include the enzymatic and the non-enzymatic antioxidants (outlined in **Table 2.2**). Enzymatic antioxidants form the body's first line of defence against oxidative stress by preventing the formation of ROS and include primarily the glutathione-dependent enzymes (Chaudiere et al. 1999) such as the glutathione reductase and the glutathione peroxidase (GPx), enzymes responsible for maintaining the balance between the oxidised and reduced forms of glutathione, respectively. Other antioxidant enzymes include the superoxide dismutases (SOD1 and SOD2) which are responsible for the dismutation of the superoxide anion (O_2^-) to H_2O_2 which is then further detoxified by glutathione peroxidase (GPx) or catalase (CAT), (**Figure 2.2**).

Non-enzymatic antioxidants, also known as free radical scavengers or chain-breaking antioxidants such as vitamin E and C, terminate the propagation of free radical generation in the electron transport chain (Noguchi et a. 2000) by donating a proton atom (H^+) to the free radical in order to saturate the unpaired electron present in their outer orbit (Halliwell 1991, Green et al. 1997, Behl et al. 1995, 1997).

2.3.1 Estrogen as an antioxidant

Estrogen is thought to exert antioxidant properties either directly by acting as a free radical scavenger due to the presence of the hydroxyl group at the C3 position of the phenolic A ring as has been demonstrated in neuronal cells (Behl et al. 1995, 1997; Green et al. 1997, Miranda et al. 2000) or indirectly by modulating the expression of antioxidant enzymes such as glutathione peroxidase (GPx4) in the oviducts (Lapointe et al. 2005).

Furthermore, estrogen-deficient animals have been characterised by the presence of intense oxidative stress associated with the lack of estrogen in different tissues (Montilla et al. 2000, Persky et al. 2000, Hernandez et al. 2000) such as liver (Ha et al. 2004), heart and brain (Ozgonul et al. 2003) indicating that the presence of estrogen might be related to the maintenance of the redox balance in those tissues.

These studies indicate that estrogen exerts direct or indirect antioxidant properties further increasing the repertoire of estrogen's beneficial actions in the body. The importance of these properties in bone is supported by studies investigating the impact of oxidative stress on bone in the absence of estrogen.

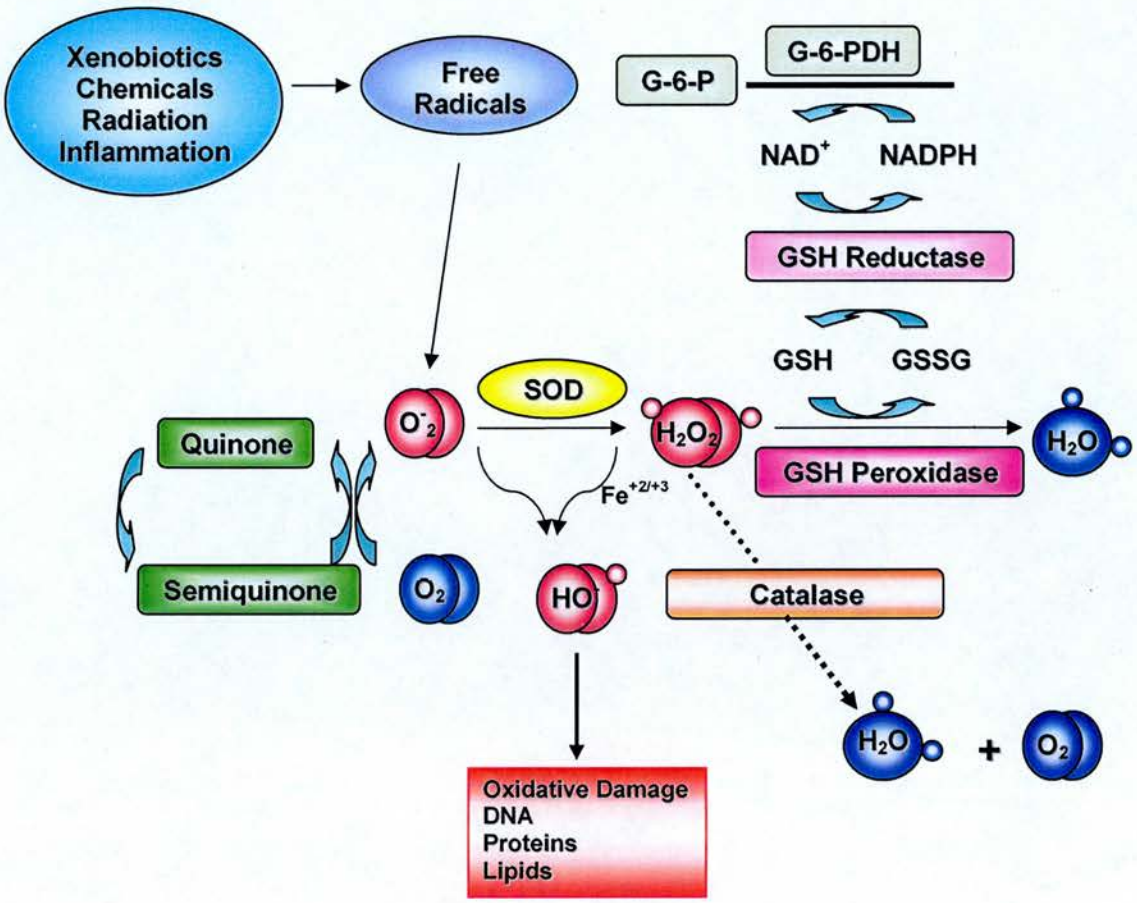


Figure 2.2. Schematic diagram of the function of the main antioxidant enzymes necessary for the cellular protection against oxidative stress.

2.3.2 Estrogen deficiency induces oxidative stress in bone

In bone tissue, estrogen deficiency and the subsequent bone loss (Allbright 1947) has been associated with accumulation of ROS resulting in a decrease in the levels of the antioxidant enzymes GPx, SOD and thioredoxin in elderly osteoporotic women (Maggio et al. 2003, Sontakke and Tare 2002), in ovariectomised rats (Isomura. et al 2004, Muthusami et al. 2005) and in ovariectomised mice (Lean et al. 2003, 2005, Jagger et al. 2005).

More direct evidence on the impact of estrogen deficiency on the accumulation of ROS such as H_2O_2 has been obtained following treatment of ovariectomised mice with catalase, which prevented ovariectomy-induced osteopenia (Lean et al. 2005). These findings indicated that the presence of ROS such as H_2O_2 is possibly engaged in mechanisms responsible for the induction of bone loss in response to estrogen deficiency. This evidence was further supported by in vitro studies that demonstrated that administration of 17β-estradiol to murine bone marrow cultures upregulated the expression of GPx in osteoclasts (Lean et al. 2005), while overexpression of GPx in the pre-osteoclastic cell line RAW cells was found to suppress osteoclast formation and differentiation (Lean et al. 2005). These findings suggest that oxidative stress might play a role in the etiology of estrogen-deficiency bone loss by creating an oxidised bone environment and point to the possible implication of estrogen in the maintenance of the redox balance in bone.

2.3.3 SERMs as antioxidants

In view of the vast amount of studies investigating the effects of estrogen as an antioxidant molecule, the action of SERMs as potent antioxidants has also been tested in several studies. For example, in neuronal cultures tamoxifen and its metabolites have been shown to decrease the propagation rate of lipid peroxidation (Wiseman et al. 1990, Moreira et al. 2004). However, tamoxifen itself has also been associated with ROS production as past of tamoxifen's mechanism to induce pro-apoptotic effects on breast

cancer cells (Mandlecar et al. 2000) and leukemic cells (Hayon et al. 1999) indicating controversial evidence for the ability of tamoxifen to act as an antioxidant in bone.

Studies have suggested that treatment with raloxifene exerts beneficial effects on endothelial dysfunction; a prerequisite of atherosclerosis characterised by enhanced release of ROS and reduced bioavailability of nitric oxide (NO). In addition, raloxifene has been shown to reduce the release of free radicals from the vascular cells by exerting antioxidant properties that improve bioavailability of NO (Wassmann et al. 2002) and possibly reduce the incidence of atherosclerosis (Wassmann et al. 2002).

2.4 Summary

Bone loss due to estrogen deficiency has been associated with increased accumulation of ROS (Lean et al. 2003, 2005). This stresses the requirement of further studies on the antioxidant properties exerted by the drugs administered to prevent and treat postmenopausal osteoporosis such as estrogen and SERMs both on the cellular and molecular level. Despite evidence suggesting the induction of osteocyte apoptosis during estrogen loss, no evidence exists so far to characterise the impact of ROS on osteocyte apoptosis. In chapters 4 and 5 of this thesis an attempt is made to study the mechanism of estrogen and SERM action against H_2O_2-induced oxidative stress in osteocytic cultures.

Enzymatic antioxidants	Cellular localization and properties
Superoxide dismutase (SOD) (SOD1, SOD2)	• Present in mitochondria, cytosol (McCord et al. 1969) • Degrades superoxide into H_2O_2 and O_2
Glutathione peroxidase (GPx)	• Present in mitochondria, cytosol, nucleus, peroxisomes (Godeas et al. 1994) • Reduces H_2O_2 and lipid peroxides
Catalase (CAT)	• Present in peroxisomes and cytosol • Decomposes H_2O_2 to H_2O
Major non-enzymatic antioxidants	**Source and function**
Glutathione (GSH)	• Present in plasma, saliva (McCord et al. 1969) • Chain breaking antioxidant or plays a role in GSH peroxidase and GSH catalase enzymatic reactions (see §2.4.1)
Vitamin E (a-tocopherol)	• Diet-derived; found in plasma, saliva (McCord et al. 1969) • Lipophilic, in all cell membranes (Leibovitz & Siegel 1980) • Chain-breaking antioxidant (Halliwell 1991)
Vitamin C	• Diet-derived • Hydrophilic • Chain breaking antioxidant (Wefers et al. 1988) • Recycles vitamin E (Halliwell 1991, Jacob 1995) and glutathione (Jacob 1995)
Selenium	• Diet-derived • Activates glutathione peroxidase (GPx) • (Leibovitz &Siegel 1980)
α-Lipoic acid	• Found in the body, diet-derived (Packer et al. 1997) • Scavenges most radicals and reduces lipid peroxidation • Recycles vitamin C
Ubiquinone	• Present in mitochondria (Ernster & Dallner 1995) • Ubiquinol (reduced form) inhibits lipid peroxidation in membranes (Stocker et al. 1991) • Prevents protein and DNA oxidation
Carotenoids (Vitamin A)	• Lipid-soluble, found in plasma (McCord et al. 1969) • Free radical scavenger
Melatonin	• Gland hormone, readily absorbed • Scavenges hydroxyl, superoxide and lipid peroxide radicals (Reiter et al. 1996) • Protects against lipid peroxidation, radiation and protein damage
Estrogen	• Systemic steroid hormone • Lipophilic • Functions are summarised in Chapter 1

Table 2.2 Enzymatic and non-enzymatic physiological antioxidant defence mechanisms.

CHAPTER 3

Selective Estrogen Receptor Modulator (SERM) Inhibits Osteocyte Apoptosis During Abrupt Estrogen Withdrawal. Implications for Bone Quality Maintenance.

Abstract

Estrogens have previously been shown to exert positive effects on the quantity and quality of bone including the maintenance of osteocytes through the inhibition of their apoptotic cell death. Selective Estrogen Receptor Modulators (SERMs) have been designed to confer all of the positive bone-associated effects of estrogens without any of their negative soft tissue side effects. This study has investigated whether the osteocyte-sparing effect of E_2 (17β-estradiol) can be mimicked by the SERM LY 117018, a Raloxifene analogue, in a rat model of ovariectomy (OVX).

Twenty-four juvenile female rats were divided into four treatment groups; sham operated (SHAM) (n=3), ovariectomy OVX (n=3), ovariectomy + 17β-estradiol (OVX +E_2) (0.125 mg/kg/day) (n=3) and ovariectomy + SERM (OVX + SERM) (3 mg/kg/day) (n=3) at two time points (n=24). Radius and ulna were removed; snap frozen and examined histologically at 7 or 14 days following the start of treatment. In the region of secondary spongiosa with cortical medullary distinction, osteocytes were examined for viability using the lactate dehydrogenase enzyme activity assay (LDH assay) and for apoptosis using an in situ nick-translation (NT) technique. The percentage of apoptotic osteocytes was significantly increased (2.5 fold at 7 days and 6 fold at 14 days) in the OVX group compared to the SHAM operated group in both the radii and the ulnae. Treatment of OVX animals with either 17β-estradiol (OVX+E_2) or LY 117018 (OVX+SERM) for 7 or 14 days following surgery prevented the increase in osteocyte apoptosis induced by OVX in both the radius and ulna.

These observations demonstrated that the LY 117018 SERM can exert a powerful inhibitory effect upon osteocyte apoptosis following estrogen loss in a similar way to the known effect of 17β-estradiol pointing to the potential benefits of SERMs on both the quantity and quality of bone in E_2 depleted rats.

3.1 Introduction

Estrogen withdrawal either due to menopause or ovariectomy has been characterized by bone loss (Allbright et al. 1941, Wronski et al. 1989, Kalu et al. 1991) and a high prevalence of osteocyte death (Tomkinson et al. 1997; 1998, Kousteni et al. 2001). The presence of osteocytes within bone has been associated with its ability to respond to mechanical stimuli (Lanyon et al. 1993), remodel efficiently and repair accumulated microdamage (Burr et al. 1985, Parfitt 2001, Noble et al. 2003) indicating that bone quality is closely associated with the presence of osteocytes and the communicating network they form through their cytoplasmic processes within the bone matrix (Dunstan et al. 1993).

Previous studies have identified an increase in the proportion of osteocytes undergoing apoptosis in ovariectomised rats (Tomkinson et al. 1998) and in women undergoing estrogen suppression when treated for endometriosis with gonadotrophin-releasing hormone (GnRH) analogs (Tomkinson et al. 1997). In these studies, estrogen administration, as part of HRT, was shown to inhibit the apoptotic death of osteocytes, hence potentially maintaining a viable osteocytic network and positively affecting bone health (Tomkinson et al. 1997, 1998). However, besides the beneficial effects of HRT on bone (§1.11.6), the chronic administration of estrogen has also been associated with an increased risk of endometrial (Grady et al 1995) and breast cancer (Colditz et al 1995). These findings have pointed to the need for the design of new molecules that would both be efficiently used for the treatment of postmenopausal osteoporosis and demonstrate tissue selective activities (McDonnell et al. 2000, Sandberg et al. 2002).

Selective Estrogen Receptor Modulators (SERMs) are currently the focus of much research effort since they have been shown to mimic the positive effects of estrogen on bone and cholesterol metabolism, without being associated with its negative effects on the reproductive tissues (Kauffman et al. 1995). Therefore, during the last decade raloxifene has effectively replaced estrogen as a first-line treatment against postmenopausal osteoporosis (Delmas et al. 1997). The LY 117018 SERM, employed in this study, is a structural analogue of raloxifene and has been shown to

engender positive effects on estrogen-deficient bone. For example, a number of studies have demonstrated that LY 117018 maintains bone mineral density in oophorectomised rats (Curiel et al. 1998, Hodsman et al. 1999) and reduces the increased bone turnover associated with estrogen deficiency, as evidenced by the reduced production of biochemical markers of bone turnover (Curiel et al. 1998). However, the effects of SERMs on the osteocytic population in bone have not been investigated so far, even though osteocytes have previously been shown to be affected by estrogen loss in ovariectomised rats (Tomkinson et al. 1998) and in women undergoing estrogen suppression with GnRH analogs (Tomkinson et al. 1997).

The rat model of OVX is a widely accepted and very frequently practiced in vivo model for investigating the pathogenesis, prevention and treatment of postmenopausal bone loss (Frost and Jee 1992), since it is characterised by changes that mimic the response of the human skeleton to estrogen deficiency. Most studies have demonstrated that ovariectomy results in an increased rate of bone turnover and trabecular bone loss followed by loss in cortical bone in rats (Wronski et al. 1988).

This study has investigated whether the use of the LY 117018 SERM could mimic the protective effects of 17β-estradiol on the maintenance of osteocyte viability and prevent the increase in the proportion of osteocytes undergoing apoptosis in a rat model of ovariectomy.

In order to detect changes in osteocyte viability methods to detect both osteocyte apoptosis and cell membrane integrity were employed. Apoptotic osteocytes were identified by the use of the Nick Translation (NT) technique (Noble et al. 1997) (§3.2.2), while viable osteocytes at the time of sampling were determined by using the Lactate Dehydrogenase Assay (LDH) (Noble et al. 2003), (§3.2.3).

3.2 Materials and Methods

3.2.1 Animals

Twenty-four, weight matched (range 250–270 g), female Sprague-Dawley rats were purchased (Charles River, Margate, UK), a minimum of seven days before the start of the experiment. A standard rodent diet (RM1; Special Diet Services Ltd, UK) and tap water were available *ad libitum*. Rats were randomised into four treatment groups and each treatment group was subdivided to provide two time points for analysis (7 and 14 days), such that each sub-group contained three animals. At Day 0, each of the animals was anaesthetised. Three of the treatment groups were ovariectomised (OVX) and an osmotic pump was implanted subcutaneously for targeted delivery to the tissue (Alzet, USA). The pump delivered one of three treatments: 17β-estradiol (OVX + E_2) (Calbiochem, UK) at a dose rate of 0.125 mg/kg/day; SERM LY 117018 (OVX + SERM) (Eli Lilly & Co., USA) at a dose rate of 3 mg/kg/day; or vehicle alone (OVX+VEH) (20% β-hydroxy-cyclodextrin). The control group was subjected to sham ovariectomy and an osmotic pump was implanted to deliver vehicle alone (SHAM + VEH) (20% β-hydroxycyclodextrin).The 4^{th} (control) group was subjected to SHAM ovariectomy and an osmotic pump was implanted to deliver vehicle alone (SHAM+VEH).

After either 7 or 14 days, as dictated by the experimental group, the animals were killed. The radius and the ulna were harvested, briefly immersed in 5% polyvinyl alcohol (Sigma, UK) and chilled to –70°C in supercooled hexane (BDH, UK), prior to storage and analysis. Animal experimentation was conducted in compliance with national ethical guidelines. Cryostat transverse sections of 7 μm thickness were cut from the chilled material and transferred to Superfrost ® Plus slides (Western Laboratory Services, UK) in order to aid adhesion of the bone section to the slide. In this study, bone sections were taken from the metaphysis. Metaphysis has been shown to be responsive to OVX by becoming osteopenic in comparison to the diaphysis which is not characterised by bone loss in OVX rats (Kimmel and Wronski 1990), (**Figure 3.1**). Three sections were used per biopsy in order to determine each of the measurement criteria. These sections were spaced at least three sections apart to avoid sampling the same osteocyte nucleus twice.

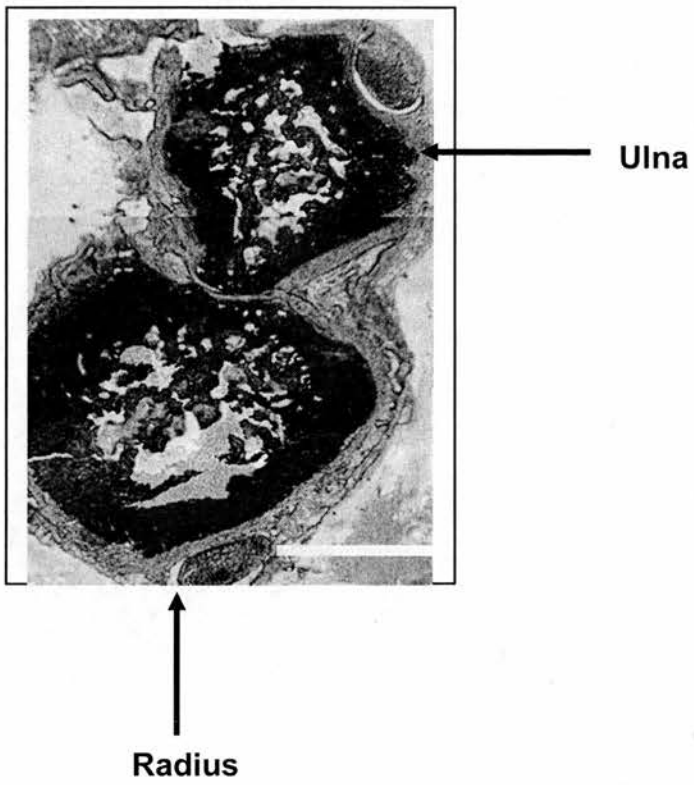


Figure 3.1. Transverse section of the rat radius and ulna. Representative image of a transverse section of the rat radius and ulna viewed under light microscopy (x 4). The image demonstrates the region of secondary spongiosa with cortical medullary distinction from which the sections used in the analysis were obtained. Bar represents 100 μm.

3.2.2 In situ analysis of osteocyte apoptosis using Nick Translation technique

The percentage of osteocytes demonstrating significant levels of DNA fragmentation was determined using a DNA nick translation technique, appropriately modified in order to be of moderate sensitivity in order to increase specificity (Noble et al. 1995; 1997). The increase in specificity of this method was achieved by reducing the polymerase concentration such that only the larger number of single DNA breaks, associated with apoptosis, relative to necrosis, were detected. This technique allows the determination of early DNA breaks, prior to the loss of any DNA content and prior to plasma membrane permeabilisation (Petit et al. 1995).

7 μm transverse cryostat sections of bone were transferred to charged Superfrost microscope slides and were fixed in 4% paraformaldehyde (Sigma, UK) for 10 minutes, washed in phosphate buffer saline (PBS) (Oxoid, UK) before demineralisation in 0.25 mol/L ethylenediamine tetra-acetate (EDTA) (Sigma, UK) in 50 mmol/L Tris HCl, pH 7.4, for 10 minutes. The sections were then washed thoroughly in PBS and air dried. One group of sections were treated with deoxyribonuclease I (DNase) (0.2mg/ml in PBS) (Sigma, UK) for 30 minutes to induce DNA breaks in order to provide a positive control of the technique. These sections were then washed in PBS (**Figure 3.2**). All sections were then treated with the nick translation mixture which consisted of 3 mmol/L digoxigenin (DIG)-labelled dUTP (DIG-11-dUTP); 3 mmol/L each of dGTP, dATP, and dCTP; 50 mmol/L Tris HCl, pH 7.5; 5 mmol/L $MgCl_2$, 0.1 mmol/L dithiothreitol, 0.5 mL/100 mL DNA polymerase I for 45 minutes at 37 ^{0}C in a humified chamber. Negative control sections were treated as above, in the absence of DNA polymerase I (**Figure 3.3**).

All sections were washed and incubated with fluorescein isothiocyanate (FITC)-labelled anti-DIG antibody (all reagents obtained from Roche Diagnostics Corporation, UK) and 5% normal sheep serum (Sigma) in PBS, for 1 hour in a humified chamber at room temperature (RT). After washing in PBS, sections were stained for nuclear DNA with propidium iodide (PI) (Sigma, UK) at 2.5 ng/ml for 3 minutes, washed thoroughly in water and mounted with fluorescent mounting

medium (DAKO, UK). Sections were then visualised by fluorescence microscopy. Osteocytes stained positive for both the FITC label and PI were considered as osteocytes containing fragmented DNA.

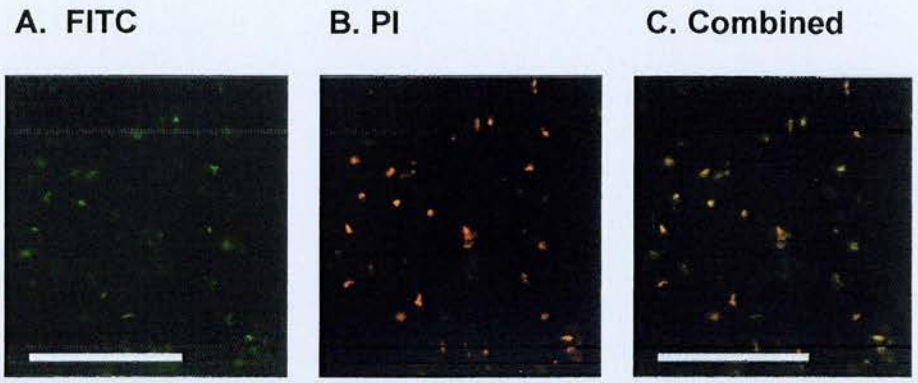


Figure 3.2. In situ demonstration of a positive control for the nick translation method. A section of cortical bone was taken from the radius of a SHAM animal. All nuclei stained green for the FITC signal **(A, C)** since the section was incubated with deoxyribonuclease I (DNase) in order to produce DNA breaks prior to reaction with the nick translation mixture (x20). Nuclei were stained red with PI **(B, C)**. Bar represents 100 μm.

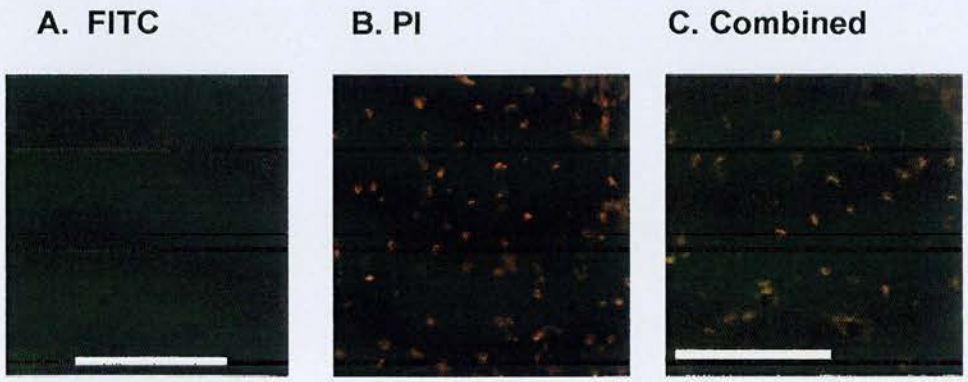


Figure 3.3. In situ demonstration of a negative control for the nick translation method. Sections were treated with the nick translation mixture without the presence of DNA polymerase I. Nuclei were stained red with PI **(B, C).** No FITC signal was observed indicating the inability of oligonucleotides to be incorporated into the DNA breaks in the absence of DNA polymerase I and be recognised by the anti-DIC-FITC labelled antibody **(A, C)** (x 20). Bar represents 100 μm.

3.2.3 Cell viability assessment in situ

One parameter used as a basis for cell viability is the metabolic activity of the viable cells. Viable cells at the time of sampling were identified in cryostat sections by means of their lactate dehydrogenase (LDH) activity. Lactate dehydrogenase is a cytoplasmic enzyme which catalyzes the oxidation of L-lactate to pyruvate by reducing nicotinamide adenine dinucleotide (NAD^+) to NADH (Stryer L. 1995).

$$\textbf{L-lactate} + \textbf{NAD}^+ \xrightleftharpoons{\textbf{LDH}} \textbf{Pyruvate} + \textbf{NADH} + \textbf{H}^+$$

Histochemical staining was undertaken using the method of Noble and Stevens (Noble and Stevens 2003). 7μm unfixed tissue sections were incubated in 1.75 mg/ml disodium salt (a-nicotinamide adenine dinucleotide, NAD) (Roche Diagnostics Corporation, UK), 60 mmol/L lactic acid, 3 mg/ml nitroblue tetrazolium (NBT) (Sigma, UK) and 40% Polypep (Sigma, UK), pH 8.0, for 3h at 37 °C in a humidified chamber. Finally, sections were rinsed in warm water to remove the reaction mixture, fixed in 4% paraformaldehyde for 10 minutes, washed in PBS and then mounted in DPX mounting medium (Fisher Scientific Ltd, UK). Sections were examined under transmitted light and the number of dark blue stained LDH positive osteocytes, considered to be viable per bone area (mm^2), was quantified using the NIH Image Analysis Software.

The metabolic activity of the cells was assessed by their capacity to convert NBT to a coloured product since the colour is the outcome of metabolic activity (Widholm, 1972). The proton atom (H^+) released during the reduction of NAD^+ to NADH reduces NBT to give a dark blue coloured product only in the metabolically active cells with intact cell membranes. This suggests that the LDH assay is an appropriate method for determining cell viability.

3.2.4 Quantification of osteocyte viability and apoptotic criteria

Transverse cryostat sections of 7 μm in thickness were obtained from the region of the secondary spongiosa with cortical medullary distinction moving in the direction of the diaphysis for 2.5mm, approximately 0.6 mm from the base of the growth plate. (**Figure 3.1**). Three non-consecutive sections from each bone were used to calculate the percentage of apoptotic osteocytes by estimating the number of apoptotic osteocytes using the nick translation technique relative to the total number of cells which were stained positive with propidium iodide (PI). Adjacent sections from the same area were used to determine the density of viable osteocytes (the number of LDH positive osteocytes per mm^2) using the *in situ* LDH viability assay. All analyses in this study were blinded. A minimum of nine non-overlapping fields of view were randomly selected from each section so that generally more than 70 % of the total bone area in each section was analysed using NIH image analysis software. In all cases, a total of at least 1000 osteocytes were assessed per bone.

The following criteria were used in order to calculate the percentage of apoptotic osteocytes and the density of viable osteocytes following individual treatments:

Percentage of apoptotic osteocytes (%) =

$$\frac{\text{Average number of FITC positive osteocytes}}{\text{Average number of PI stained osteocytes}} \times 100$$

Density of viable osteocytes (mm^{-2}) =

$$\frac{\text{The number of LDH positive osteocytes}}{\text{The area of bone in the field of measurement per } mm^2}$$

In this study, sections were analysed as 2D structures and therefore the density measurement represents an "areal density" (mm^{-2}), (Parfitt et al. 1987) rather than volume based density (mm^{-3}) of osteocytes. However, due to the fact that sections have a specific thickness only the osteocytes on a particular plane of focus were counted in each field of view.

It should be noted that the percentage of viable osteocytes in each section (the number of LDH positive osteocytes divided by the total number of osteocytes stained with PI) was not quantified in this study since, in some cases, the number of LDH positive osteocytes exceeded the total number of osteocytes (PI stained) assessed in the bone section. Taking into account that that the section thickness was 7μm while the dimension of an osteocyte is about 10-20 μm (Cooper et al. 1966, McCreadie et al. 2004), this finding might be explained by the fact that osteocytes could have been cut through their cytoplasm (LDH positive osteocytes) without reaching their nuclear DNA required in order for them to be quantified as PI positive osteocytes.

3.2.5 Statistical analysis

All statistical analyses were performed using quantitative data analysis with SPSS release 11.5 for Windows. Data was checked for normal distribution by applying the Kolmogorov-Smirnov test. In cases where the randomly selected sample data were shown to have a normal (Gaussian) distribution (95% of data would fall within plus or minus 1.96 standard deviations from the mean value) parametric statistical tests such as two-tailed Analysis of Variance (ANOVA) and the post-hoc Tukey-Kramer test and Dunnett test were performed directly to determine statistical significance between the treatment groups. For comparison between percentages of osteocyte apoptosis used in this study, the square root of each percentage was transformed into its arcsine, which enabled the distribution of the data to be nearly normal allowing therefore the use of parametric tests. Normal distribution of the data allowed the use of parametric tests such as Analysis of Variance (ANOVA) followed by Dunnett's post hoc test (when the p value of the ANOVA was $p<0.05$) in order to allow comparison of all treatment groups against the control group (SHAM) (Zar 1984,

treatment groups as it allows for comparison of more than two means without introducing the type I error associated with multiple t-tests (Zar 1984, Fielding and Gilbert 2000). Results are expressed as means ± S.E. $p<0.05$ was considered to be statistically significant.

3.3 Results

3.3.1 Ovariectomy induces osteocyte apoptosis in both the radius and ulna

Seven days after surgery, OVX led to a significant 2.5-fold increase in the percentage of apoptotic osteocytes compared to SHAM operated rats in both the radius (1.27% vs. 0.45%, respectively; p= 0.02) **(Figure 3.4A, Figure 3.6)** and the ulna (1.05% vs. 0.40%, respectively; p=0.015) **(Figure 3.4B)**, as estimated using the nick translation technique. 14 days after OVX, osteocyte apoptosis was increased to 6-fold compared to SHAM treated rats in both the radius (1.78% vs. 0.27%, respectively; p= 0.01) **(Figure 3.5A)** and the ulna (1.4% vs. 025%, respectively; $p < 0.001$) **(Figure 3.5B)**.

3.3.2 17β-estradiol prevents ovariectomy-induced osteocyte apoptosis in both the radius and ulna

Slow release of 17β-estradiol (OVX+E_2) at a dose rate of 0.125 mg/kg/day for 7 days following OVX reduced the percentage of OVX-induced apoptotic osteocytes compared to OVX treatment, in both the radius (0.41% vs 1.27% respectively, p=0.014) (**Figure 3.4A**) and the ulna (0.36% vs 1.05% respectively, p=0.01) (**Figure 3.4B**) to levels similar to SHAM for both the radius and the ulna (p>0.05), (**Figures 3.4A and 3.4B**).

In addition, application of 17β-estradiol (E_2) for 14 days following OVX also prevented the increase in apoptotic osteocytes due to OVX treatment in both the radius (0.38% vs 1.78% respectively, p=0.02) (**Figure 3.5A**) and the ulna (0.68% vs 1.4% respectively, p=0.013) (**Figure 3.5B**).

3.3.3 LY 117018 SERM protects osteocytes from ovariectomy-induced apoptosis

Slow release of the LY 117018 SERM at a rate of 3 mg/kg/day for 7 days was effective in protecting the osteocytes from OVX-induced apoptosis in both the radius (0.63% vs 1.27% respectively) (**Figure 3.4A**) and the ulna (0.53% vs 1.05% respectively, p<0.05) **(Figure 3.4B)**. The proportion of apoptotic osteocytes due to

OVX was also decreased following treatment with the LY 117018 SERM both in the radius (0.23% vs 1.78% respectively, p=0.009) **(Figure 3.5A)** and the ulna (0.16% vs1.4% respectively, p<0.001), **(Figure 3.5B)**. Interestingly, suppression of OVX-engendered increase in osteocyte apoptosis by the LY 117018 SERM was significantly greater than that of 17β-estradiol in the ulna (0.16% vs 0.68%, respectively, p=0.004) 14 days following ovariectomy **(Figure 3.5B)**.

3.3.5 Osteocyte viability

As also noted in previous studies (Tomkinson et al., 1998), ovariectomy in this study did not affect the proportion of viable osteocytes either in the radius or the ulna after 7 or 14 days relative to SHAM (p>0.05 in all cases), **(Figures 3.7 and 3.8), (Figure 3.10)**. Treatment with 17β-estradiol or LY 117018 had no effect on the number of viable osteocytes at 7 or 14 days post-operation, in either of the two bones compared to SHAM animals (**Figure 3.7 and 3.8**).

3.3.6 % Change in body weight.

Animals were weighed prior to treatment and regularly thereafter. As shown in **Table 3.1**, no statistically significant difference was observed in the percentage change in body weight at 7 days following ovariectomy. However, by day 14 post-surgery, comparison of the body weights prior to and following treatment revealed significant differences between the experimental groups (p<0.05). Therefore, the percentage change in body weight following different treatments was identified. After 14 days of surgery, OVX rats were shown to have an increase in the percentage rate of body weight gain compared to SHAM (17.21% ± 1.89.vs 5.74% ± 0.63, p=0.003). Treatment with either 17β-estradiol or LY 117018 for 14 days significantly reduced the percentage increase in body weight in OVX rats to SHAM levels or below (-0.34 ± 1.45 and 1.75 ± 0.74 vs 5.74% ± 0.63), (p= 0.035).

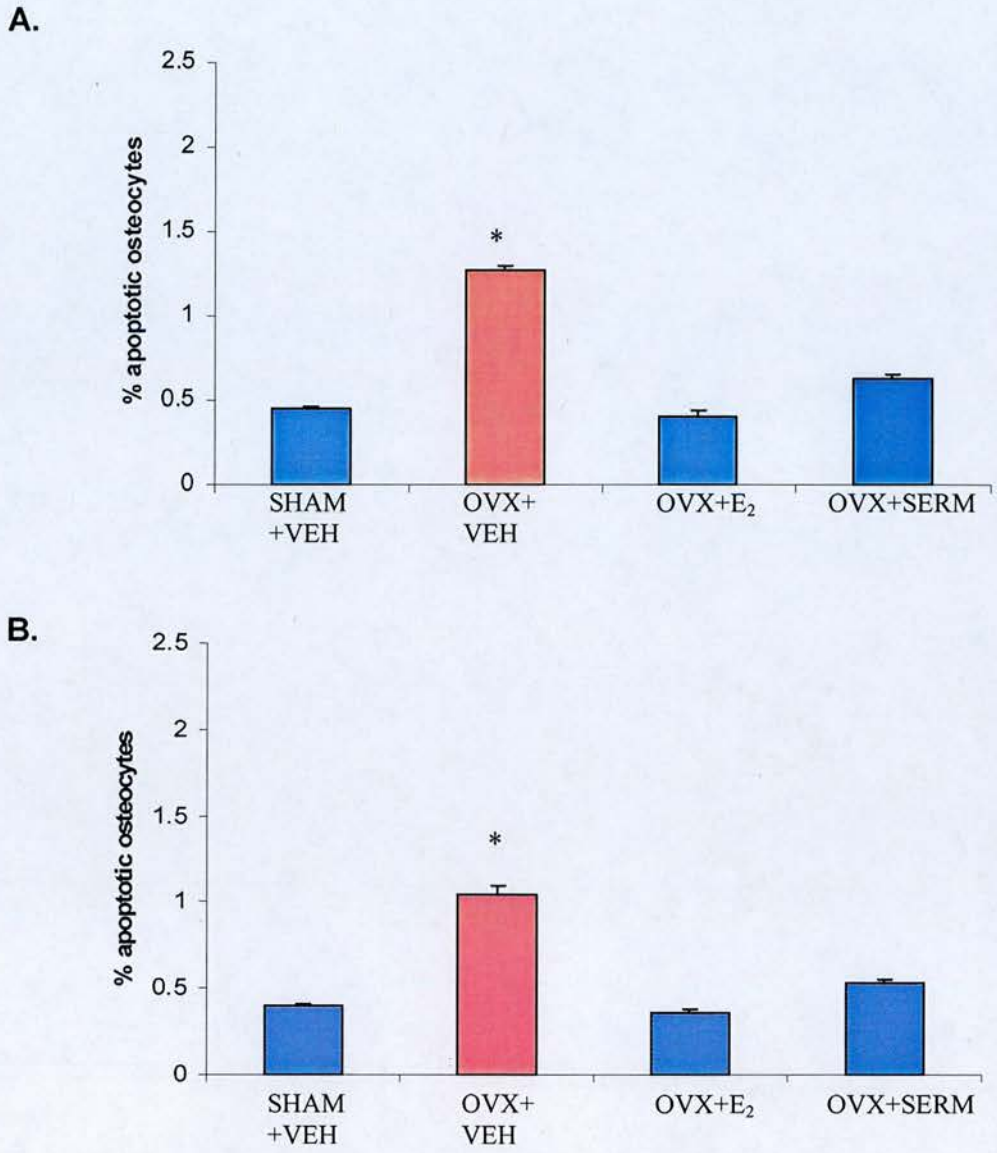


Figure 3.4. The percentage of osteocytes displaying evidence of DNA fragmentation in situ after 7 days. 17β-estradiol and LY 117018 SERM prevented OVX-induced osteocyte apoptosis in situ after 7 days of treatment in both the **A.** radius and **B.** ulna. Sections obtained from all the treatment groups were studied for the presence of fragmented DNA material using an in situ nick translation technique. Results are expressed as the mean percentage of total osteocytes displaying positive staining for fragmented DNA. ± S.E. *$p<0.05$ relative to the SHAM control.

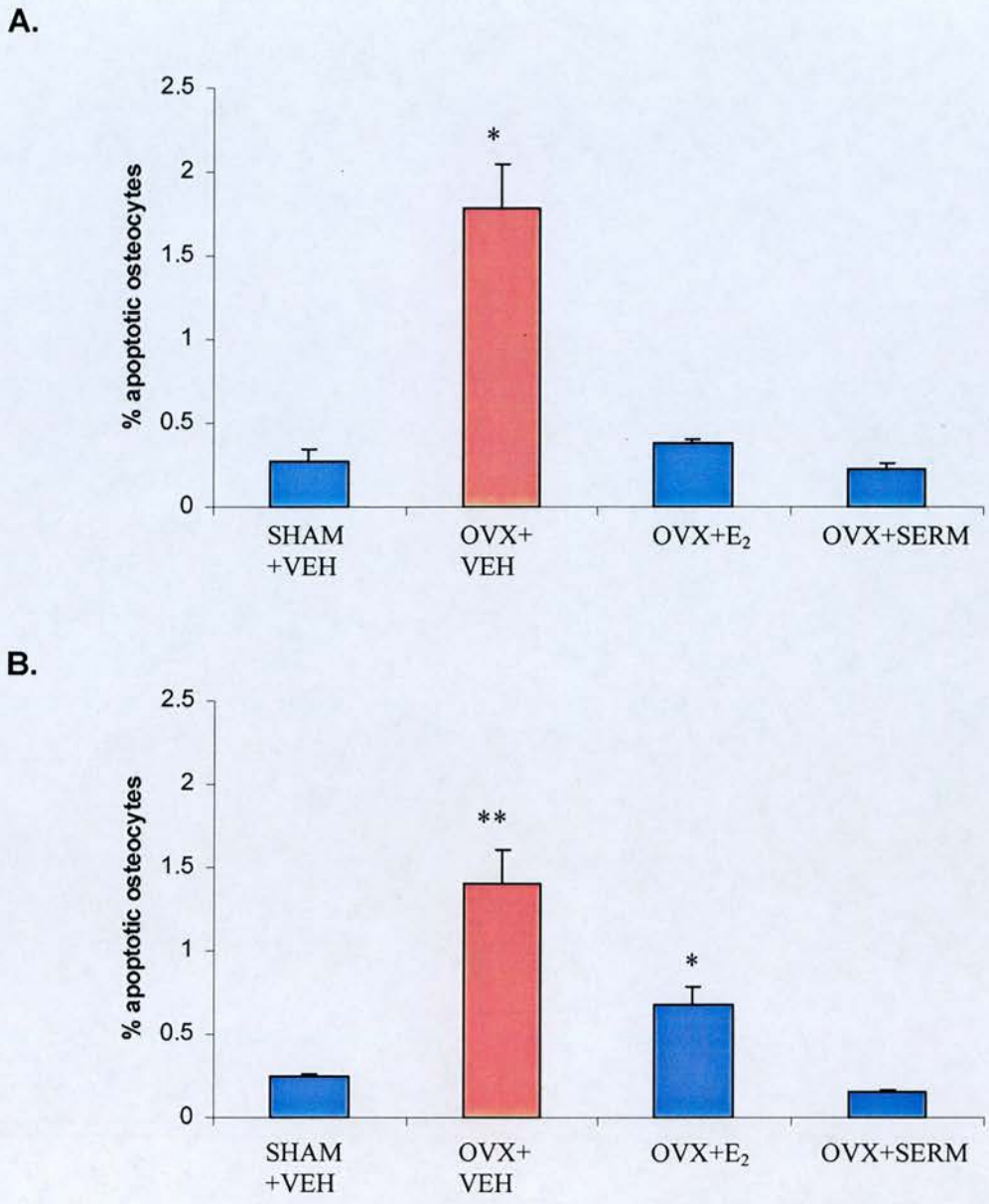


Figure 3.5. The percentage of osteocytes displaying evidence of DNA fragmentation in situ after 14 days. 17β-estradiol and LY 117018 SERM prevented OVX-induced osteocyte apoptosis in situ after 14 days of treatment. Sections were obtained from the **A.** radius and **B.** ulna and studied for the presence of fragmented DNA material using an in situ nick translation technique. Results are expressed as the mean percentage of total osteocytes displaying positive staining for fragmented DNA. ± S.E. *$p<0.05$, ** $p<0.001$ relative to the SHAM control.

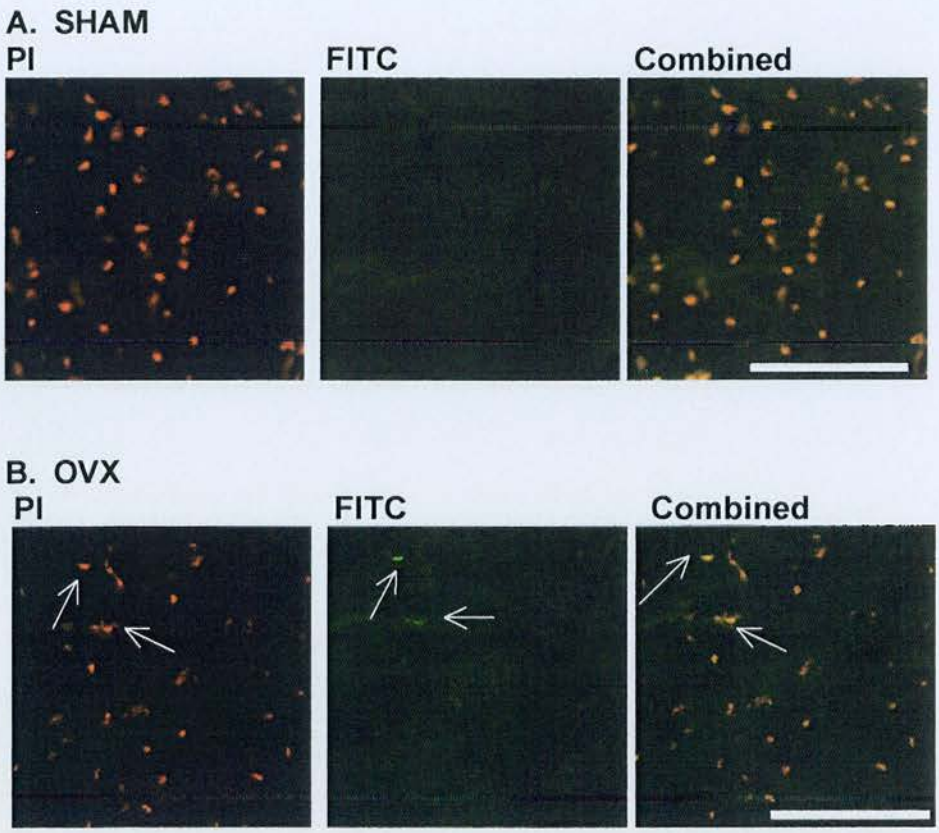
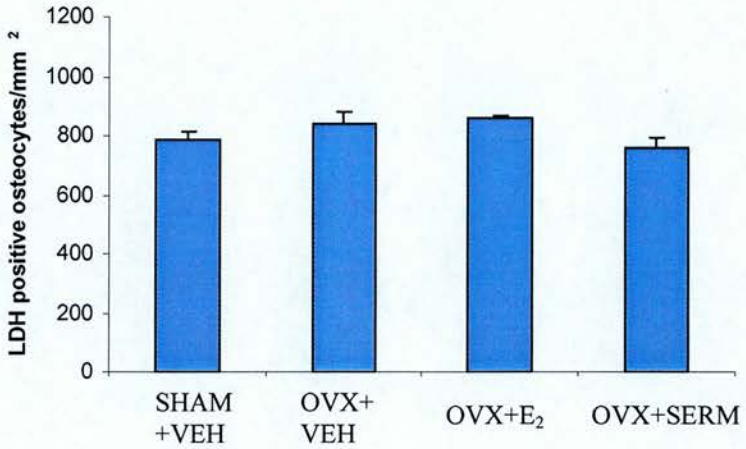


Figure 3.6. In situ demonstration of osteocytes containing fragmented nuclear DNA in response to OVX. Sections obtained from **A.** SHAM and **B.** OVX animals were reacted with the NT mixture and counterstained with PI (red) to identify nuclear DNA. White arrows **(B)** illustrate osteocytes staining positive for DNA fragmentation (FITC-green), identified as an orange signal where FITC-green signal was co-localised with PI (red) nuclear staining (x20). Bar represents 100 μm.

A.



B.

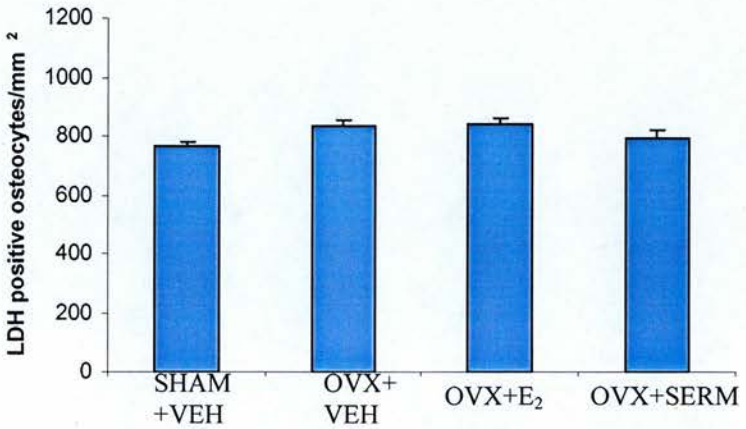
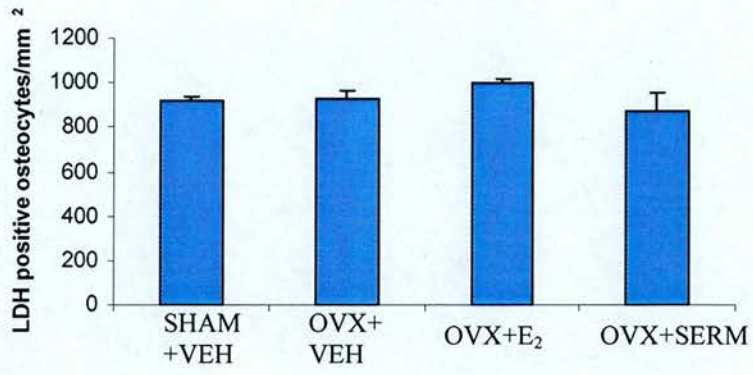


Figure 3.7. The density of LDH positive osteocytes 7 days post-operation. The number of viable osteocytes (LDH positive cells) per bone area (mm^2) 7 days post-operation in both the **A.** radius and **B.** ulna was not altered by the treatments. Sections were studied for the presence of cellular LDH activity and results are expressed as the mean ratio of the number of LDH positive osteocytes per area (mm^2) for every treatment.

A.



B.

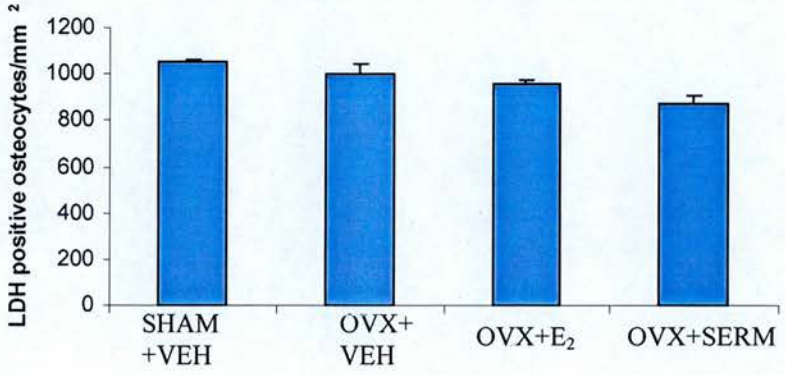


Figure 3.8. The density of LDH positive osteocytes 14 days post-operation. The number of viable osteocytes (LDH positive osteocytes) per bone area (mm^2) 14 days post-operation in both the **A.** radius and **B.** ulna is not altered by the treatments. Sections were studied for the presence of cellular LDH activity and results are expressed as the mean ratio of the number of LDH positive osteocytes per area (mm^2) for every treatment.

Treatments (n=3)	Percentage change in body weight	
	DAY 7	DAY 14
SHAM/VEH	3.40 ± 0.63	5.74 ± 0.63
OVX/VEH	7.61 ± 0.59	17.21 ± 1.89*
OVX/E_2 (0.125mg/kg/day)	-4.48 ± 1.13	-0.34 ± 1.45*
OVX/SERM (3mg/kg/day)	2.83 ± 2.08	1.75 ± 0.74*

Table 3.1. Effects of 17β-estradiol and LY 117018 on body weight in the OVX rat following 7 and 14 days of treatment. Results are expressed as group means of body weight ± S.E. * represents $p<0.05$ relative to the SHAM control at day 14.

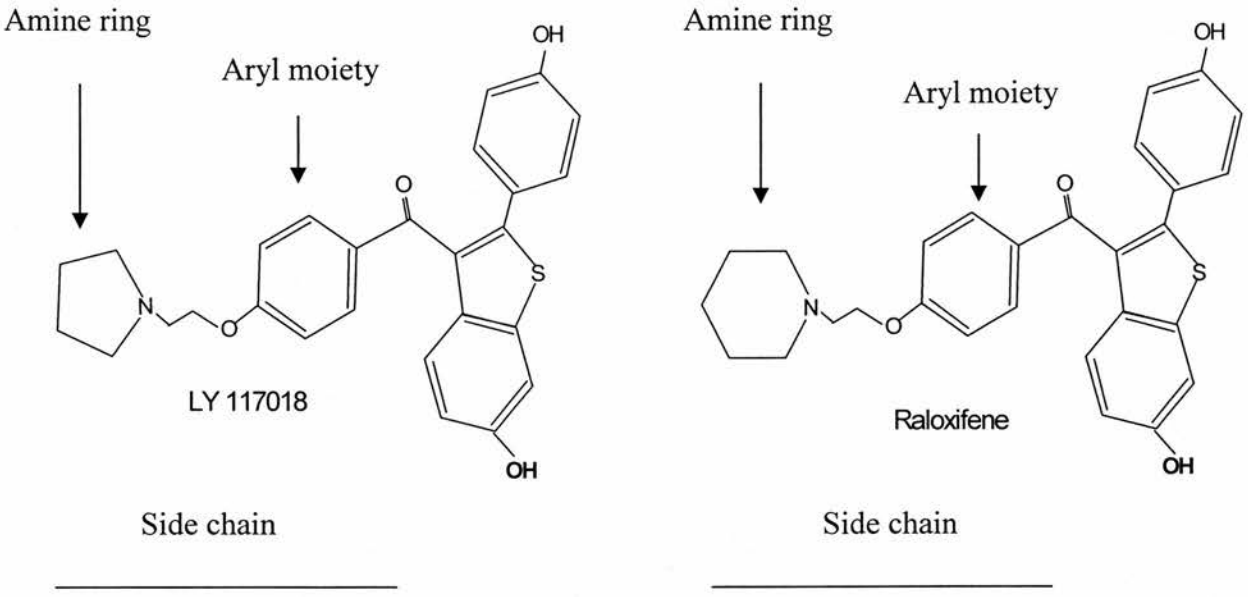


Figure 3.9. Basic chemical structures of LY 117018 and Raloxifene.

LY 117018 is characterised by the presence of a non-steroidal core and a basic side chain of an aryl moiety and an amine ring. The side chain of the LY 117018 SERM is similar to that of raloxifene, lacking however one carbon atom at the amine ring.

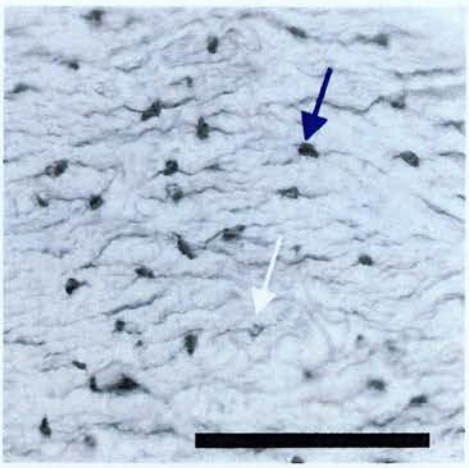


Figure 3.10. Histochemical reaction of the LDH enzyme in rat sections in situ. Representative image of a section obtained from an OVX animal and stained for LDH activity. Viable osteocytes were distinguished by the presence of LDH activity in cells. Blue arrow identifies a viable LDH-positive osteocyte. White arrow points to lacunae which were excluded from measurement (x20). Bar represents 100μm.

3.4 Discussion

The study presented in this chapter has provided evidence that, in a rat model of estrogen loss, administration of the LY 117018 SERM mimicked the protective effects of 17β-estradiol against osteocyte apoptosis associated with ovariectomy.

Although the beneficial effects of SERMs on bone have been well characterised on the tissue level and have been long used in bone as a part of HRT, little is known so far regarding the effects of SERMs on bone cells and especially on the osteocytic population. According to the concept of osteocytes being the mechanosensors and mechanotransducers in bone (Pead et al. 1988, Skerry et al. 1989, El Haj et al. 1990, Lanyon 1993; Burger et al. 1995), osteocytes sense changes in the amount and direction of mechanical loading and signal the need for removal of microdamage (Burr et al. 1985, Parfitt. 2001, Noble et al. 2003). Based on these findings suggesting that the presence of osteocytes within bone is crucial for the maintenance of mechanically optimal bone microarchitecture, impaired osteocyte viability could potentially result in lack of bone mass and poor bone quality. Studies have shown that osteocyte cell death in response to ageing, osteoporosis (Mullender et al. 2005) and osteoarthritis lead to increased bone fragility (Frost 1960, Dunstan et al. 1990, 1993; Qiu et al. 2003) possibly due to loss of the ability of bone to sense or repair microdamage (Parfitt 1993).

Osteocytes have previously been shown to be negatively affected by estrogen loss in human, rat (Tomkinson et al. 1997, 1998) and mouse tissue (Kousteni et al. 2001). Removal of estrogen by GnRH treatment in humans or by OVX in rats resulted in a substantial increase in the number of osteocytes undergoing apoptosis (400% and 375%, respectively), as evidenced by the in situ nick translation technique at 24 and 3 weeks, respectively (Tomkinson et al. 1997, 1998). In this study the time points of 7 and 14 days were selected based on previous experience on osteocyte apoptosis in these bones (Noble et al. 2003). Although the detailed time course of the estrogen loss-engendered osteocyte apoptosis in vivo is unknown, in this study apoptotic osteocytes in both the radius and the ulna were identified as early as 7 days following ovariectomy and were increased by 182% and 162% respectively, based on

identification of DNA fragmentation using an in situ nick translation technique (Noble et al. 1997).

In this study, administration of LY 117018, a structural analogue of raloxifene, at the known bone sparing dose of 3mg/kg/day (Bowman et al. 1996, Hodsman et al. 1999) resulted in the protection of osteocytes from OVX-induced apoptosis in both the radius and ulna 7 and 14 days following surgery. Administration of 17β-estradiol (0.125 mg/kg/day) was shown to inhibit the osteocyte apoptosis induced by 21 days of OVX (Tomkinson et al. 1998). In this study, the same dose of 17β-estradiol was employed, based on the study by Tomkinson et al., and was shown to prevent osteocyte apoptosis at the earlier time points of 7 and 14 days post-surgery. These findings confirm the importance of estrogen in the maintenance of the osteocytic population in bone. However, investigation of more time points-including the 21 day time point-would have allowed the direct comparison regarding the anti-apoptotic effects of 17β-estradiol between these studies.

In agreement with previous studies (Tomkinson et al. 1998), the increase in osteocyte apoptosis observed in this study due to ovariectomy in the rat was not associated with gross changes in the number of viable osteocytes compared to control that was estimated to be in the range of 800-1000 osteocytes per mm^2 and was found to be similar to the mean value of ~ 800 osteocytes/mm^2 reported by Tomkinson et al. (Tomkinson et al. 1998*)*. This finding could be explained by the fact that apoptotic cells remain viable and maintain membrane integrity until the early stages of apoptosis (Wyllie et al. 1980) providing further evidence supporting the apoptotic nature of osteocyte death in this model. An alternative explanation might take into account the rapid rate of remodelling thought to occur in rats (Tomkinson et al. 1998) which might have contributed to the efficient renewal of dead or dying cells. However, in contrast to rodent bone, the more slowly turning over human bone has been characterised by a cumulative loss of osteocytes as a result of increased osteocyte apoptosis associated with estrogen loss (Tomkinson et al. 1997) indicating that the rate of bone remodelling plays an important role in osteocyte viability. However, the effects of ovariectomy on osteocyte apoptosis in this study are small

(<2%) based on the low incidence of apoptosis observed in these samples. The small percentage of apoptotic osteocytes might also explain the lack of change in the density of viable osteocytes.

As expected, there was a significant increase in body weight following ovariectomy that manifest at 14 days of treatment (Wronski et al. 1987). Previous studies have indicated that estrogen loss in both women (Gambacciani et al. 1997) and rats (Wronski et al. 1987) is associated with a significant gain in body weight. An increase in fat mass has two obvious confounding influences on bone metabolism; firstly the estrogenic nature of fat and secondly the conferring increased mechanical load on some components of the skeleton. Both of these influences would be predicted to decrease osteocyte apoptosis based on our current understanding (Noble et al. 2003). The fact that osteocyte apoptosis is influenced upon OVX is likely to be the consequence of the overpowering influence of estrogen loss compared to the very small weight gains observed in these studies. The fact that administration of estrogen reduced body weight to below the SHAM levels could suggest that the dose of estrogen employed in this study was possibly high. Possible over-dosage could have been investigated by measuring the weights of the uteri. In agreement with previous studies showing that the LY 117018 compound is capable of inhibiting weight gain (Bowman et al. 1996, Li et al. 1998, Curiel et al. 1998), administration of LY 117018 to OVX-treated animals reduced the OVX-induced increase in the body weight to levels lower than that in SHAM animals.

The precise anti-apoptotic mechanism of action employed by these compounds was not addressed in this study. In vitro studies by Kousteni et al have suggested that sex steroids exert non-genotrophic receptor-dependent anti-apoptotic effects on osteoblasts and osteocytes involving the activation of a mitogen-activated protein signalling pathway (Kousteni et al. 2001, 2003). Alternatively, data in Chapters 4 and 5 have demonstrated that both 17β-estradiol and LY 117018 are capable of preventing osteocyte apoptosis possibly in a non-genomic, receptor-independent manner by exerting antioxidant activities on osteocytes.

In conclusion, treatment with the SERM LY 117018 inhibited the increase in osteocyte apoptosis associated with estrogen loss mimicking the bone-sparing effects of 17β-estradiol in a rodent model of ovariectomy. The present study is the first to elucidate that current therapies for the treatment of osteoporosis, such as SERMs, exert positive estrogen-like effects on bone viability at the cellular level by maintaining the osteocytic population following ovariectomy.

In addition, there is growing interest in the current hypothesis that osteocytes are important in maintaining bone quality and modulating bone formation (possibly through sclerostin secretion) (Poole et al. 2005) and resorption (Noble et al. 2003). This study highlights an important bioactivity that may explain part of the action of SERMs in vivo. While the effect of LY 117018 on bone quality has not been directly addressed in this study, a number of other studies have demonstrated that administration of LY 117018 to OVX rats exert clear bone-sparing effects, as evidenced by a reduction in the increase in bone turnover due to OVX and improvement of BMD (Bowman et al. 1996, Li et al. 1998, Curiel et al. 1998, Hodsman et al. 1999). It is possible that the effects of SERMs on preventing osteocyte apoptosis will in the future be harnessed to increase their effectiveness in the treatment of a number of clinical conditions including those of old age.

SECTION 2

Inhibition of osteocyte apoptosis

CHAPTER 4

The anti-oxidant effect of 17β-estradiol in the inhibition of osteocyte apoptosis *in vitro*.

Abstract

This work is the subject of a published manuscript. Estrogen withdrawal promotes postmenopausal bone loss and is associated with the apoptotic death of osteocytes *in vivo* (Tomkinson et al. 1997, Tomkinson et al. 1998). Although administration of HRT in the form of 17β-estradiol has demonstrated clear estrogen receptor-mediated benefits to bone mass, the molecular mechanism by which estrogen maintains bone cells has not been fully elucidated. A large number of studies have documented protective effects of estrogen against oxidative stress in neuronal and endothelial cells. However, no studies have investigated the effects of estrogen against oxidative stress induced in bone cells. The purpose of this study was to examine whether 17β-estradiol could benefit the osteocytic population by exerting antioxidant properties against oxidative stress-engendered apoptotic cell death in the osteocyte-like MLO-Y4 cell line.

MLO-Y4 osteocytes were treated with estrogen and estrogen derivatives alone or in combination with the specific estrogen receptor antagonist ICI 182,780 prior to the induction of oxidative stress with H_2O_2. The effects of estrogenic compounds on oxidative stress induced by H_2O_2 were studied by quantification of apoptosis and ROS generation in osteocytic cultures.

H_2O_2 was shown to induce osteocyte apoptosis, which was significantly inhibited in the presence of 17β-estradiol. Similar protective effects of 17β-estradiol were also observed in ex vivo calvarial osteocytes. However, the receptor antagonist ICI 182,780 did not abrogate the effect of 17β-estradiol possibly implying an estrogen receptor-independent action. In addition, ICI 182,780 also demonstrated protective effects on its own against H_2O_2-induced apoptotic death stimuli. Use of the stereoisomer 17α-estradiol that binds weakly to the estrogen receptor also prevented H_2O_2-induced apoptosis in MLO-Y4 osteocytes. Furthermore, pre-treatment of the ER-negative HeLa and HEK 293 cell lines with 17β-estradiol in the presence of H_2O_2 prevented oxidative stress-induced death, further supporting a possible estrogen receptor-independent effect of 17β-estradiol. Common to all of the five compounds with anti-apoptotic effects tested in this study was

the presence of the putative free radical scavenger C3-OH moiety on the steroid A-ring. The two compounds mestranol and quinestrol, which lack the C3-OH group, did not prevent oxidant induced apoptosis. In addition, all C3-OH containing compounds, but not mestranol nor quinestrol, significantly reduced intracellular ROS generation in the presence of H_2O_2, in MLO-Y4, HeLa and HEK 293 cell lines, as measured by the use of the H_2DCF-DA dye.

These data have suggested that the anti-apoptotic effects of 17β-estradiol on MLO-Y4 osteocytes, HeLa and HEK 293 cells might operate through a possible antioxidant mechanism of action and could aid the understanding of the potential use of 17β-estradiol and estrogen-related compounds in the improvement of both the quantity and quality of bone through osteocyte saving.

4.1 Introduction

The presence of viable osteocytes within bone has been associated with the bones' ability to remodel efficiently (Kamijou et al. 1994, Power et al. 2001, Qiu et al. 2002, Marotti et al. 1998, Hernandez et al. 2004) and to repair the accumulated microdamage (Frost et al. 1998, Burr et al. 1993). The number of viable osteocytes has been shown to decrease during the process of ageing (Frost 1960, Dunstan et al. 1993), while increased osteocyte apoptosis has been associated with chronic administration of glucocorticoids (Weinstein et al. 1998, 2000), estrogen loss (Tomkinson et al. 1997, 1998), fatigue microdamage (Verborgt et al. 2000, Noble et al. 2003) and disuse (Bakker et al. 2004, Basso et al. 2006, Aguirre et al. 2006); indicating that the maintenance of a viable osteocytic population in bone is an important parameter of bone quality. Estrogen loss has been shown to increase osteocyte apoptosis in human and rat bone (Tomkinson et al. 1997; 1998), while the ovariectomy-induced stimulation of osteocyte apoptosis in rats was shown to be reversed following estrogen administration (Tomkinson et al. 1998).

The mechanisms by which estrogen exerts its effects are broadly categorized as genomic when they involve gene transcription mediated through the binding of estrogen to the intracellular estrogen receptors (Razandi et al. 1999, Tsai et al. 1998) or as non-genomic (Pietras & Szego 1975; 1977, Wehling et al. 1997, Collins & Webb 1999, Levin et al. 1999, Falkenstein et al. 2000) when estrogen binds to estrogen receptors located in the plasma membrane activating intracellular signaling pathways such as the MAPK pathway (Kousteni et al. 2001) and the PI3K (Levin et al. 2002). For example, estrogen was shown to exert non-genomic anti-apoptotic effects against death stimuli induced by dexamethasone, TNF-α (Kousteni et al. 2001, 2003) and etoposide administration through the activation of the ERK1/2 pathway in osteocyte cultures in vitro (Kousteni et al. 2001, 2003).

In addition, there is evidence accumulating that estrogen could also act as a free radical scavenger through the presence of the hydroxyl group (-C3OH group) at the C3 position of the phenolic A ring (-OH group) of the hormone, as has been for example

demonstrated in neuronal cell cultures where estrogen has been shown to prevent oxidative stress-induced cell death (Behl et al. 1995, 1997). Several in vivo studies have also suggested a beneficial role of estrogen administration in the development of Alzheimer's disease (AD) in postmenopausal women (Tang et al. 1996), a disease that has been strongly associated with oxidative stress (Behl et al. 1992, 1994; Yan et al. 1996), further supporting the possible action of estrogen as an antioxidant molecule.

Previous studies have shown that administration of the antioxidant enzyme catalase, which prevents the accumulation of H_2O_2, to ovariectomised mice reversed bone loss induced by ovariectomy in mice (Lean et al. 2005) while treatment with H_2O_2 has been shown to induce apoptosis in MLO-Y4 osteocytes in vitro (Kikuyama et al. 2002). However, no evidence is available so far to demonstrate possible antioxidant effects of estrogen on osteocytes against oxidant attack.

The purpose of this study was to investigate whether estrogen could act as an antioxidant molecule against oxidant-induced death of MLO-Y4 osteocytes in culture. The ER antagonist ICI 182, 780 and the ERK inhibitor UO 126 were used to demonstrate possible involvement of the estrogen receptors and the ERK ½ pathway, respectively. In addition, various estrogen derivatives that contain the C3-OH moiety in their structure were used to indicate free radical scavenging activity in the protective effects of estrogen against oxidant-induced apoptotic cell death.

4.2 Materials and Methods

All chemicals were purchased from Sigma-Aldrich, UK, unless otherwise stated. All tissue culture reagents were purchased from Invitrogen, UK and tissue culture well plates were purchased from Corning, UK. Tissue culture procedures were performed in a laminar flow hood (class 2) receiving HEPA-filtered air, using sterile equipment.

4.2.1 Cell culture and maintenance

The murine long-bone derived MLO-Y4 osteocyte-like cell line (obtained from Prof. Lynda Bonewald, School of Dentistry, University of Missouri-Kansas City) was cultured in Modified Eagles Medium Alpha (α-MEM) supplemented with 5% fetal bovine serum (FBS), 5% newborn calf serum (NCS), 1% penicillin/streptomycin (P/S) and 1% L-glutamine (Kato et al. 1997). The HeLa human cervical epithelial cell line (obtained from the European Collection of Cell Cultures) was cultured in α-MEM supplemented with 5% FBS, 5% NCS, 1% P/S, and 1% L-glutamine (Kousteni et al. 2001). The HEK 293 human embryonic kidney epithelial cell line (obtained from Prof Philippa Saunders, MRC Reproduction Unit, University of Edinburgh) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % FBS, 1% P/S, and 1% L-glutamine (Evdokiou et al. 1999).

Growth media were stored at 4°C and warmed to 37°C prior to use. Cells were maintained in an incubator at 37°C in a 95% humidified atmosphere of O_2:CO_2 in the ratio of 95%: 5%. Sub culturing was performed once weekly upon reaching 90% of confluency, maintaining the cells in the log phase of proliferation. The monolayer of cells was detached following the addition of 1 ml of trypsin solution at 2.5 gm/l for 5-10 minutes, followed by addition of 10 ml of growth medium. The MLO-Y4 cell suspension was diluted 1:10 in fresh growth media in 75cm^2 tissue culture flasks while the HeLa and the HEK 293 cell suspensions were diluted 1:10 in fresh growth media in 25 cm^2 sterile tissue culture flasks. Tissue culture plastics, either in the form of flasks or well plates, used to maintain the MLO-Y4 cell line were coated with 0.1M collagen type I from rat tail prior to use.

4.2.2 Cell treatment

MLO-Y4 osteocytes and HeLa cells were plated at a density of 2.4 x 10^4 cells per ml and HEK 293 cells were plated at a density of 5 x 10^4 cells per ml in 24 multi-well plates, 24 hours prior to experimentation. Following appropriate treatment, cells were returned to the incubator at 37 ^{0}C for 2 hours. Experiments were carried out a minimum of three times, and each treatment group was represented by 3 wells in each experiment. Cells were observed in 3 fields per well resulting in 9 fields per treatment group using a x 20 magnification lens for all estimates allowing similar numbers of cells to be counted per field (approximately 40-100 cells per field) in all individual experiments using an inverted microscope fitted with DXM 1200 camera (Nikon UK Limited). All estrogenic compounds were diluted in 70 % ethanol while H_2O_2 was directly diluted in growth medium. Control treatments represent untreated cultures. Where indicated in the figure legends, vehicle represents treatment with 70 % ethanol subjected to dilutions identical to the highest concentration under study in the absence of the compound.

4.2.3 Induction of oxidative stress

MLO-Y4 osteocytes and HeLa cells were incubated in growth medium supplemented with H_2O_2 within a concentration range of 0.08mM-0.6mM for 2 hours in order to induce oxidative stress. HEK 293 cells were treated with H_2O_2 at the concentration of 0.3 mM previously shown to induce oxidative stress in this cell line (Nomura et al. 2006). The induction of apoptotic cell death was monitored microscopically using nuclear morphological criteria.

4.2.4 Prevention of cell death and oxidative stress

All agents that were used to prevent cell death remained in culture during H_2O_2 induced oxidative stress.

4.2.4.1 Estrogens

MLO-Y4 osteocytes and HeLa cells

MLOY-4 osteocytes and HeLa cells were pre-treated for 1 hour with 17β-estradiol (Calbiochem, UK) and the biologically inactive stereoisomer 17α-estradiol (Sigma)

(**Figure 4.12**) diluted in 70% ethanol at concentrations of 10^{-6}M to10^{-8}M (Kousteni et al. 2002), followed by H_2O_2 treatment used at 0.3mM for 2 hours.

HEK 293

HEK 293 cells were pre-treated for 1 hour with 17β-estradiol (Calbiochem, UK) diluted in 70% ethanol at concentrations of 10^{-6}M to10^{-8}M (Kousteni et al. 2002), followed by H_2O_2 treatment used at 0.3mM for 2 hours.

4.2.4.2 Estrogen-related compounds

MLO-Y4 osteocytes

MLOY-4 osteocytes were pre-treated for 1 hour with the estrogen receptor antagonist ICI 182, 780 (Wilson et al. 2000, Gu et al. 2005, Kousteni et al. 2001), (Tocris Cookson Ltd), (**Figure 4.12**) at a concentration range of 10^{-6}M-10^{-8}M, diluted in 70% ethanol, followed by H_2O_2 treatment used at 0.3mM for 2 hours.

MLO-Y4 osteocytes and HeLa cells

MLO-Y4 osteocytes and HeLa cells were pre-treated for 1 hour the estrogen derivatives ethinylestradiol 3-methyl ether (Mestranol), 17α-ethinylestradiol and ethinyl estradiol 3-cyclopentyl ether (Quinestrol) (**Figure 4.12**), diluted in 70% ethanol, at a concentration range of 10^{-5} M-10^{-10} M (Behl et al. 1997) followed by H_2O_2 treatment used at 0.3mM for 2 hours.

4.2.4.3 Vitamin E

The anti-oxidant Vitamin E was used to investigate the potential cell saving activity of a potent antioxidant compound in this model system. MLO-Y4 osteocytes were pre-treated for 1 hour with a-tocopherol (vitamin E), (**Figure 4.12**), at concentrations of 10^{-4} M, 10^{-6} M and 10^{-8} M (Behl et al. 2000), diluted in 70% ethanol, followed by H_2O_2 treatment used at 0.3mM for 2 hours.

M, 10^{-6} M and 10^{-8} M (Behl et al. 2000), diluted in 70% ethanol, followed by H_2O_2 treatment used at 0.3mM for 2 hours.

4.2.5 Inhibitors of intracellular signaling proteins

4.2.5.1 Inhibition of caspase 3/7 activity

Caspases are involved in various stages in apoptotic cascades, mediating either initial stages in death receptor pathways or events further down in apoptotic pathways that involve the disassembly of the cell's structure (Thornberry et al. 1998). To determine whether osteocyte apoptosis in response to H_2O_2 was a caspase-dependent process osteocytes were pre-incubated with the selective caspase 3/7 inhibitor (GlaxoSmithKline, USA), a non-peptide selective inhibitor of caspases 3 and 7 based on sulphanilamide derivatives (Lee et al. 2000) mainly mediating end-stage cytoskeletal and nuclear changes in the apoptotic cell at a concentration of 1 μM for 1 hour prior to 0.3mM H_2O_2 treatment.

4.2.5.2 MAPK inhibitors

U0126 is a chemically synthesized organic compound that inhibits activation of MAPK (ERK 1/2) by inhibiting the kinase activity of MAP Kinase Kinase (MAPKK or MEK 1/2) (Favata et al. 1998). Cells were pre-incubated for 1 hour with UO126 (Calbiochem, UK) at a concentration of 20μM in DMSO, a concentration previously shown to inhibit ERK in osteocytes (Kogianni et al. 2004). UO 126 remained in the culture media prior to the further addition of 17β-estradiol and estradiol derivatives, and then also subsequently in the presence of H_2O_2.

4.2.6 Determination of apoptotic state

4.2.6.1 DAPI staining for healthy and apoptotic cell morphology

The DAPI (4', 6-Diamidino-2-phenylindole) compound stains nuclear DNA, giving a fluorescent image of nuclear structure when subjected to UV light illumination at 344 nm. DAPI reveals chromatin condensation, nuclei shrinkage and the fragmentation of

the nuclear material into smaller blebs indicating late stages of the apoptotic cascades (Cowden et al. 1981). It is rapidly taken up by the cells and forms fluorescent complexes with double-stranded DNA by forming stable hydrogen bonds with the minor groove of the double helix. Following experimental treatments, cells were fixed in 4% paraformaldehyde for 10 minutes, washed three times in PBS and air-dried. Cells were then incubated with DAPI at a concentration of 2.5ng/ml for 20 minutes. Normal cells were characterised by intact nucleus and normal cytoplasmic appearance whereas apoptotic cells were characterised by cell shrinkage, plasma membrane blebbing observed using transmitted light and nuclear condensation examined using fluorescence (358nm excitation, emission 461nm). Necrotic cells were identified by cell swelling and/or bursting and loss of membrane integrity. Cell numbers were also determined following H_2O_2 treatment in order to monitor the rapid cell death characteristic of necrosis.

Within each field the total cell number and the number of apoptotic cells was assessed; the results were expressed as the percentage of the ratio of apoptotic to non-apoptotic cells, as calculated using the equation:

$$\text{Percentage of apoptotic osteocytes per field} = \frac{\text{Average number of DAPI apoptotic osteocytes per field}}{\text{Total number of DAPI stained osteocytes per field}} \times 100$$

4.2.6.2 DNA fragmentation using in situ Nick Translation

The percentage of target osteocytes demonstrating DNA breaks was investigated in murine calvaria using the in situ nick translation staining (Noble et al.1997), which allows the determination of DNA breaks following the incorporation of DIG-labelled dUTP, as described in § 3.2.2. The ratio of total osteocytes (DAPI stained) to apoptotic (FITC positive) was determined using fluorescence microscopy with digital capture.

the presence of ROS. It becomes de-esterified intracellularly and turns to a highly fluorescent 2', 7'-dichlorodihydrofluorescein upon oxidation by intracellular H_2O_2 (Bass et al. 1983, Lebel et al. 1992; Royal et al. 1993) and reactive oxygen species (Zhu et al. 1994, Gunasekar et al. 1995). H_2DCF-DA was added to the culture medium of MLO-Y4 osteocytes and HeLa cells at 10 μM (Yen. et al. 2001, Ohashi et al. 2002) for 30 minutes prior to addition of either 17β-estradiol, 17α-estradiol, ICI 182, 780, 17α-ethinyl estradiol, vitamin E, mestranol or quinestrol for 1 hour. HEK 293 cells were incubated with H_2DCF-DA for 30 minutes and then with 17β-estradiol or vitamin E for a further hour. All cultures received treatment with H_2O_2 0.3mM for a further 2 hours. The H_2DCF-DA and pre-treatment agents remained in culture during H_2O_2 treatment after which the media were removed and the cells were washed twice with PBS before re-addition of normal growth medium. Live cells were monitored using an inverted microscope fitted with an environmental chamber to maintain temperature at 37 ^{0}C and 5% humidified CO_2. ROS positive cells were detected at 490nm excitation and images captured at 20x magnification using DXM1200 colour camera. For analysis 3 wells per treatment and six fields per well were counted. Negative control was represented by cultures incubated in the absence of the H_2DCF-DA in order to determine the absence of auto-fluorescence in MLO-Y4 cultures.

4.2.8 Isolation of murine calvariae for *ex vivo* experimental conditions

Mouse calvariae were aseptically isolated from 12 Balb/c mice. Calvariae were carefully broken across the suture line in order to provide controls from each animal (for each of the treatments) and were kept in individual wells in a 24-well plate in a-MEM medium supplemented with 5% FBS, 5% NCS, 1% P/S and 1% L-glutamine. The calvariae were then randomly divided into 4 treatment groups (n=3 for each group). The treatment groups are comprised of control, H_2O_2 and 17β-estradiol in the presence or absence of H_2O_2. Calvariae were pre-treated for 1 hour with 17β-estradiol (10^{-8}M) followed by H_2O_2 treatment at 0.3 mM for 24 hours at 37 °C. Following the experimental period, the bones were submerged immediately in 5% polyvinyl alcohol and snap frozen in a hexane-chilling bath before being stored at -70 °C prior to cryostat sectioning. Cryostat

groups are comprised of control, H_2O_2 and 17β-estradiol in the presence or absence of H_2O_2. Calvariae were pre-treated for 1 hour with 17β-estradiol (10^{-8}M) followed by H_2O_2 treatment at 0.3 mM for 24 hours at 37 °C. Following the experimental period, the bones were submerged immediately in 5% polyvinyl alcohol and snap frozen in a hexane-chilling bath before being stored at -70 °C prior to cryostat sectioning. Cryostat sections of 7μm thickness were cut from the chilled material and transferred to 3-aminopropylnethoxy-silane (TESPA) coated slides, which aid adhesion of the bone section to the slide by creating a highly charged surface. Nine sections (three sections per each calvaria) were used per treatment group, scored for the total number of osteocytes inside the lacunae using nuclear counterstain (DAPI) while the percentage of apoptotic osteocytes for each treatment was estimated using the nick translation technique.

4.2.9 Statistical analysis

All statistical analyses were performed using quantitative data analysis with SPSS 11.5 for Windows. Data was checked for normal distribution by applying the Kolmogorov-Smirnov test. When the p value of the ANOVA was $p<0.05$, the use of the Tukey-Kramer post-hoc test allowed comparison of more than two means at once without introducing the type I error associated with multiple t-tests (Zar 1984, Fielding and Gilbert, 2000). Results are expressed as means ± S.E. The probability score $p<0.05$ was considered to be statistically significant. Results were considered significant when $p<0.05$ denoted by *; $p<0.01$ denoted by ** and $p<0.001$ denoted by *** when experimental treatment was compared to H_2O_2 treatment, and $p<0.05$ denoted by †; $p<0.01$ denoted by †† and $p<0.001$ denoted by ††† when experimental treatment was compared to the non treated control.

4.3 Results

4.3.1 H_2O_2 induces MLO-Y4 cell apoptosis in a dose dependent-manner

MLO-Y4 osteocytes were cultured with H_2O_2 at a range of concentrations between 0.08mM and 0.6mM for 2 hours **(Figure 4.1A)**. Healthy and degenerating (apoptotic and necrotic) cell morphology was investigated with DAPI nuclear staining, as described in the methods section. H_2O_2 induced apoptosis in osteocytes in a dose dependent manner over a 2 hour incubation period (**Figure 4.1A**). An increased proportion of apoptotic cells compared to control cultures was observed at H_2O_2 concentrations as low as 0.08mM and and reached maximal levels at H_2O_2 concentrations above 0.4mM **(Figure 4.1A)**. However, apart from apoptosis, concentrations higher than 0.4mM also induced necrotic cell death of osteocytes as confirmed by the presence of cells that had expanded and burst, characteristics of necrosis, and by the rapid reduction in cell numbers within 2 hours of addition to cultures (**Figure 4.1B**). In contrast, H_2O_2 concentrations below 0.4mM induced the morphological characteristics of apoptosis and did not alter cell numbers within 2 hours (**Figure 4.1B, Figure 4.2**). Based on the apoptotic criteria and the loss of cells in culture in response to the different concentrations investigated, the dose of 0.3mM, which did not induce necrosis and induced a moderate reduction in cell number at 2 hours, was selected for future experiments.

4.3.2 Caspase 3/7 selective inhibitor reduces H_2O_2- induced apoptosis

In order to further demonstrate the apoptotic nature of the oxidant engendered response a specific caspase 3/7 inhibitor (GlaxoSmithKline, USA), which is based on Isatin Sulphonamides (Lee et al. 2001), was used to inhibit terminal effector caspases 3 and 7. Pre-incubation of the cells for 1 hour with the specific caspase 3/7 inhibitor prior to H_2O_2 treatment at 0.3mM significantly reduced the percentage of apoptotic cells induced by H2O2 to near control levels confirming caspase dependency of osteocyte death under these conditions (**Figure 4.3**).

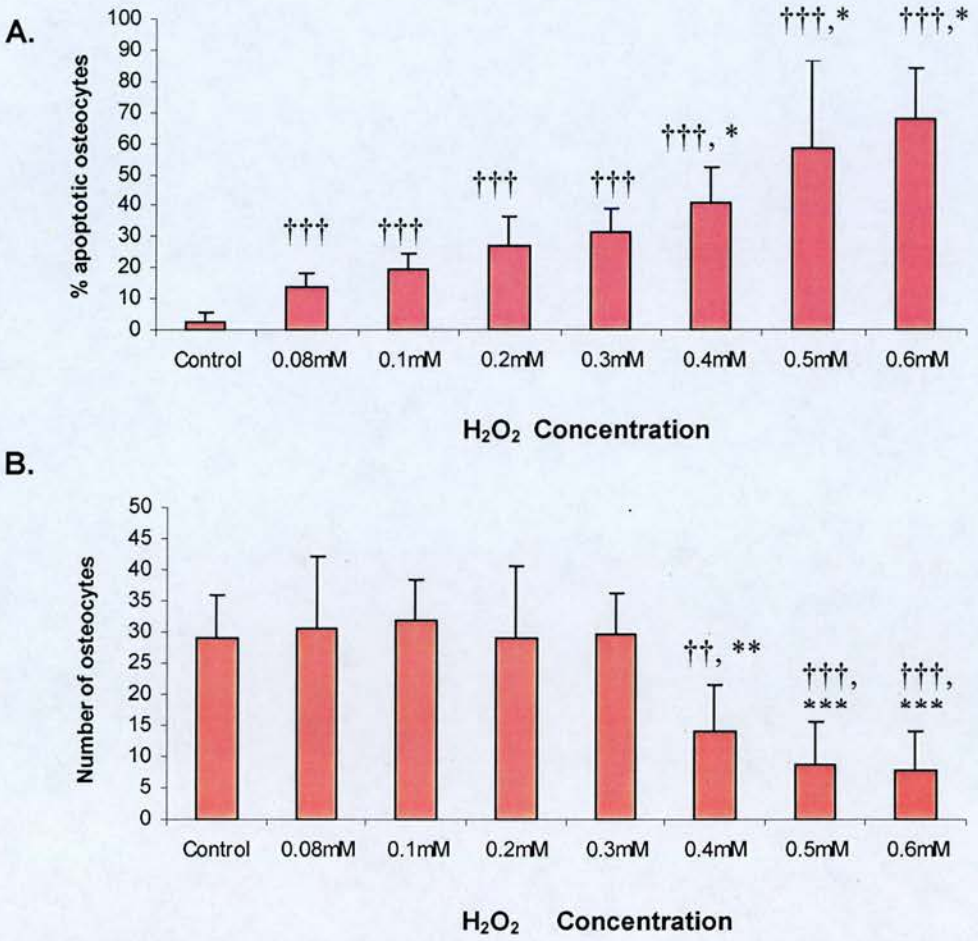


Figure 4.1. H_2O_2 induces apoptosis in MLO-Y4 osteocytes in a concentration-dependent manner. MLO-Y4 osteocytes were incubated with H_2O_2 at 0.08-0.6 mM for 2 hours. Cell were stained for apoptosis determination with DAPI and examined by fluorescent microscopy. Graphs represent **A.** mean percentages of apoptotic osteocytes ± SE. and **B.** mean number of osteocytes in culture ± SE. Control cultures represent untreated cultures. (††† = $p< 0.0001$, †† = $p< 0.0001$ relative to the untreated control, *** =$p<0.0001$, ** = $p< 0.001$, * = $p< 0.05$).

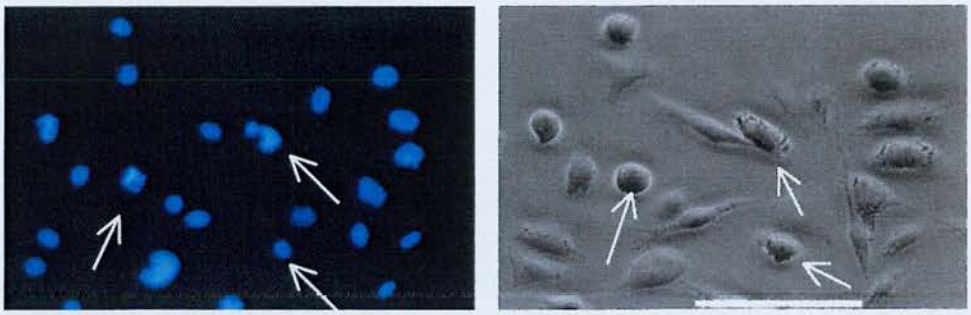


Figure 4.2. H_2O_2 induces cytoplasmic and nuclear condensation, DNA fragmentation and formation of osteocyte apoptotic bodies. Representative images of H_2O_2-treated MLO-Y4 osteocytes at H_2O_2 0.3 mM for 2 hours and stained with DAPI. Arrows indicate nuclear condensation and fragmentation (DAPI stain) and formation of apoptotic bodies (phase-contrast microscopy). Bar=10μm, 20x magnification.

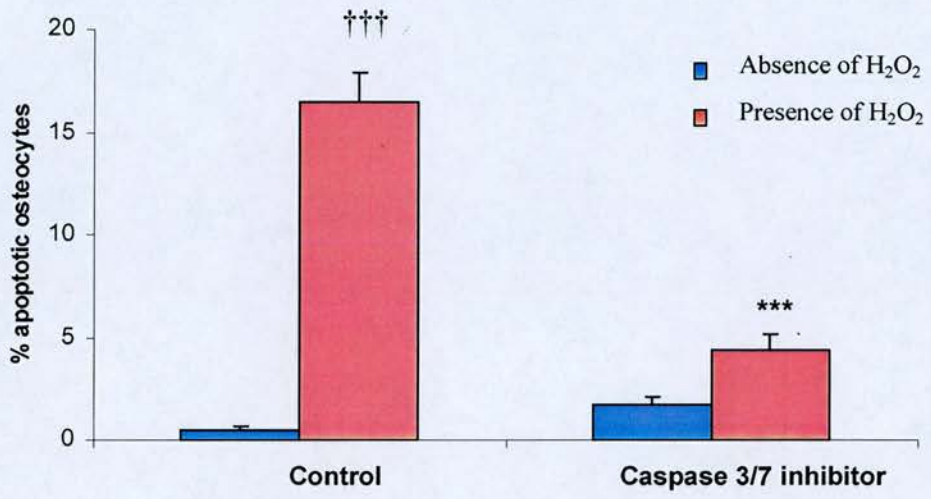


Figure 4.3. Inhibitor of caspase 3/7 reduces pro-apoptotic stimuli induced by H_2O_2. MLO-Y4 osteocytes were incubated with the selective inhibitor of **caspase 3/7** for 1 hour, prior to H_2O_2 treatment at 0.3 mM for 2 hours. Cells were stained with DAPI and examined by fluorescence microscopy. Graphs represent mean percentages of apoptotic osteocytes ± SE. (*** =$p<0.0001$ compared to H_2O_2 treatment, ††† = $p<0.0001$ compared to control). ■ = control cultures, ■ = H_2O_2 treated cultures in the absence or presence of caspase 3/7 inhibitor. Control cultures represent untreated cultures.

4.3.3 17β-estradiol prevents H_2O_2-induced apoptosis in MLO-Y4 osteocytes

Dose response studies were used to identify concentrations of 17β-estradiol that did not increase apoptosis above the control levels. Mean percentages of osteocytes displaying apoptotic morphology were significantly increased compared to the control following treatment with 17β-estradiol at 10^{-6}M ($p<0.0001$), whereas concentrations of 10^{-7}M and 10^{-8}M did not increase the levels of apoptosis above control levels ($p>0.05$) (**Figure 4.4**). 17β-estradiol was then used to treat osteocytes prior to addition of H_2O_2 to cultures. The percentage of H_2O_2 induced apoptotic osteocytes was significantly reduced after pre-treatment of cells for 1 hour with 17β-estradiol at doses of 10^{-6} M ($p<0.01$), 10^{-7} M ($p<0.007$) and 10^{-8} M ($p<0.001$) (**Figure 4.4).** The dose of 10^{-8} M, which is near the physiological concentration of 17β-estradiol (Ganong 1999, Dotsch et al. 2001), was used in subsequent treatments since it significantly reduced H_2O_2 induced apoptosis without itself demonstrating a pro-apoptotic effect on osteocytes (**Figure 4.5**).

4.3.4 17α-estradiol prevents H_2O_2-induced apoptosis in MLO-Y4 osteocytes

The pro-apoptotic stimuli by H_2O_2 in osteocytes were further investigated in the presence of 17α-estradiol, a stereoisomer of 17β-estradiol that is characterised by a lower binding affinity to estrogen receptor than 17β-estradiol and has been reported by many studies to be biologically inactive (Gorski et al. 1994). Dose response studies were used to identify concentrations of 17α-estradiol that did not increase apoptosis above the control levels. Mean percentages of osteocytes displaying apoptotic morphology were increased compared to control following treatment with 17α-estradiol at 10^{-6}M, whereas concentrations of 10^{-7}M and 10^{-8}M did not increase the levels of apoptosis above control levels ($p>0.05$) (**Figure 4.6**). Pre-treatment of cells for one hour with 17α-estradiol at concentrations of 10^{-6} M to 10^{-8} M resulted in a significant reduction in the percentage of apoptotic osteocytes induced by H_2O_2 treatment at all the concentrations used (**Figure 4.6).** The dose of 10^{-8}M was chosen for subsequent experiments using 17α-estradiol, since it reduced H_2O_2 induced osteocyte apoptosis without itself exerting toxic effects to the cells and it was similar to the minimum effective protective dose of its isomer, 17β-estradiol.

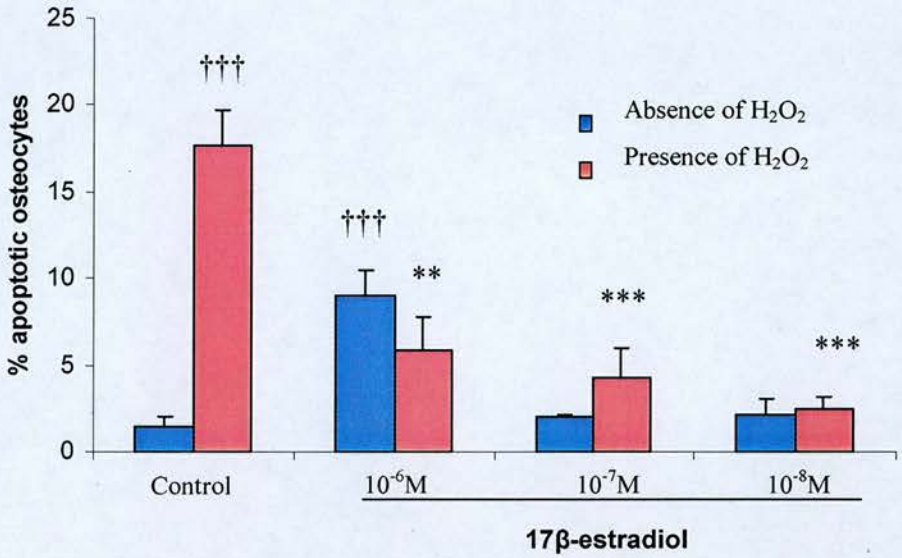


Figure 4.4. 17β-estradiol prevents MLO-Y4 osteocyte apoptosis induced by H_2O_2. Osteocytes were incubated with 17β-estradiol at 10^{-6} M, 10^{-7} M and 10^{-8} M for 1 hour prior to H_2O_2 treatment for a further 2 hours. Mean percentages of apoptotic osteocytes were statistically different compared to control following treatment with 17β-estradiol at 10^{-6}M. All concentrations of 17β-estradiol significantly reduced the percentages of apoptotic osteocytes induced by H_2O_2 treatment to levels similar to control. Cells were stained with DAPI and examined with fluorescence microscopy Graphs represent mean percentages of apoptotic osteocytes ± SE. (*** =$p<0.0001$, ** = $p< 0.001$, compared to H_2O_2 treatment, ††† = $p<0.0001$ compared to control). Control cultures represent untreated cultures. Vehicle cultures for 17β-estradiol at 10^{-6}M were similar to control in percentages of apoptotic osteocytes (8.3 ± 1.35 % S.E., $p>0.05$).

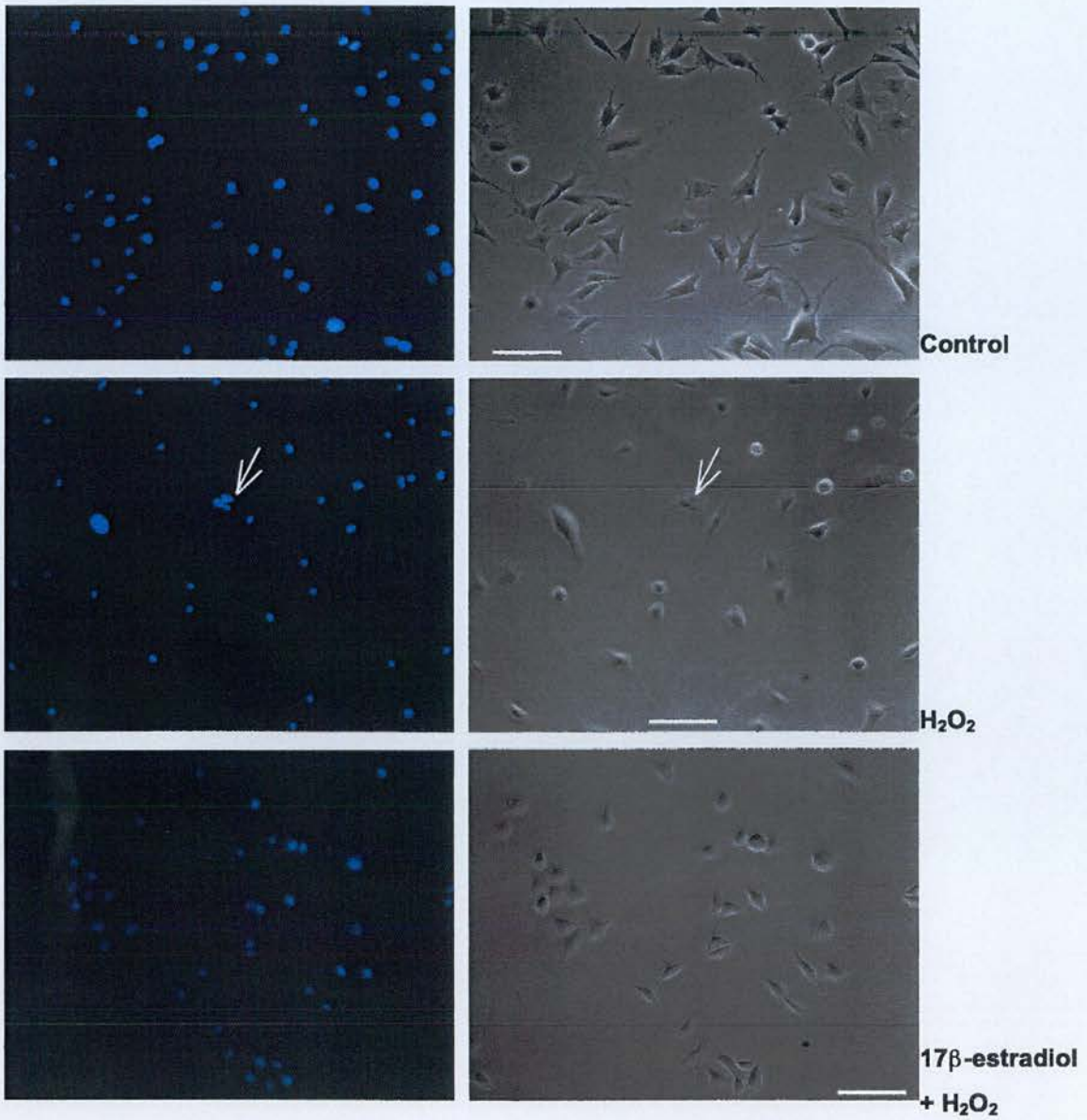


Figure 4.5. 17β-estradiol prevents H_2O_2-induced apoptosis in MLO-Y4 osteocyte cultures. Representative images of osteocytes in culture treated with 17β-estradiol for 1 hour followed by H_2O_2 treatment for a further two hours and stained with DAPI. H_2O_2 induced osteocyte apoptosis (middle panel) which was reversed following administration of 17β-estradiol (bottom panel). White arrow shows an apoptotic osteocyte (middle panel). Bar = 10 μm.

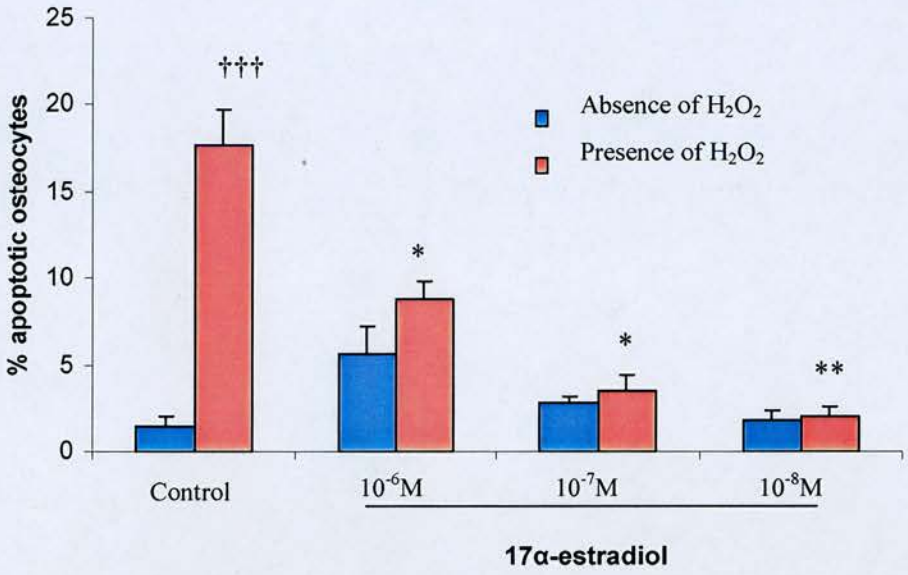


Figure 4.6. 17α-estradiol prevents MLO-Y4 osteocyte apoptosis induced by H_2O_2. Osteocytes were incubated with 17α-estradiol at 10^{-6} M, 10^{-7} M and 10^{-8} M for 1 hour prior to H_2O_2 treatment for a further 2 hours. All concentrations of 17α-estradiol significantly reduced the percentages of apoptotic osteocytes induced by H_2O_2 treatment to levels similar to control. Cells were stained with DAPI and examined with fluorescence microscopy. Graphs represent mean percentages of apoptotic osteocytes ± SE. (** = $p< 0.001$, * = $p< 0.05$ compared to H_2O_2 treatment, ††† = $p<0.0001$ compared to control). Control cultures represent untreated cultures. Vehicle cultures for 17α-estradiol at 10^{-6}M were similar to control in percentages of apoptotic osteocytes (5.3 ± 0.81 % S.E., $p>0.05$).

4.3.5 The estrogen receptor antagonist ICI 182 780 does not prevent the anti-apoptotic effects of 17β-estradiol

In order to determine whether the anti-apoptotic effects of 17β-estradiol were mediated via the nuclear estrogen receptor, the pure estrogen receptor antagonist ICI 182,780 was used. Dose response studies showed that ICI 182 780 did not prevent the anti-apoptotic effects of 17β-estradiol at any of the concentrations used in this study ($p=0.0005$ at 10^{-8}M) (**Figure 4.7A**). The lowest dose of 10^{-8} M of ICI 182 780, which is similar to the dose used for 17β-estradiol, was employed in subsequent treatments. (**Figure 4.7A**).

ICI 182 780 prevents H_2O_2-induced osteocyte apoptosis

Estimation of the apoptotic osteocyte levels induced by H_2O_2 in the presence of ICI 182 780 indicated that pre-treatment of osteocytes with the ICI 182 780 compound in the absence of 17β-estradiol was sufficient to reduce H_2O_2 pro-apoptotic stimuli ($p= 0.001$, compared to H_2O_2 treatment) (**Figure 4.7B**).

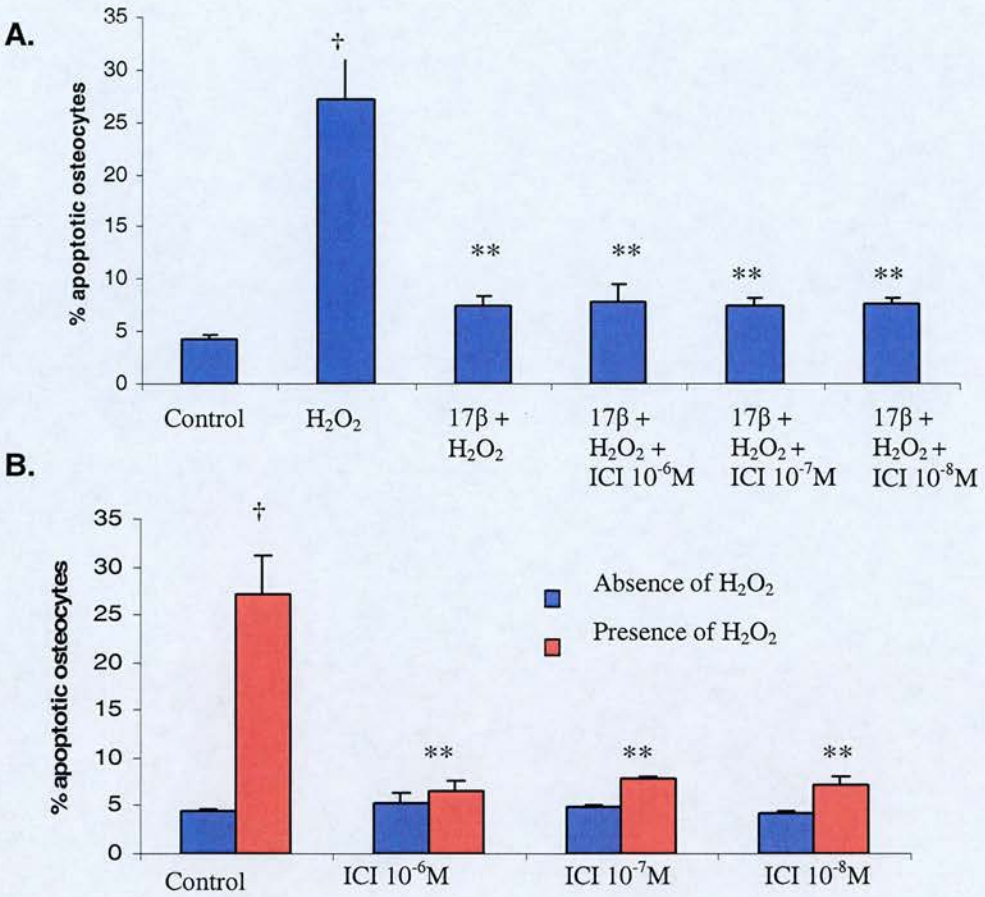


Figure 4.7. ICI 182 780 does not prevent the protective effects of 17β-estradiol on MLO-Y4 osteocyte apoptosis induced by H_2O_2. A. Osteocytes were pre-incubated with ICI 182 780 at 10^{-6} to 10^{-8} M for 1 hour prior to addition of 17β-estradiol for 1 hour and H_2O_2 treatment for a further 2 hours. ICI 182 780 did not prevent the anti-apoptotic effects exerted by 17β-estradiol. **B.** Pre-treatment of cells with ICI 182 780 alone prior to the addition of H_2O_2 prevented H_2O_2-induced osteocyte apoptosis. Cells were stained with DAPI and examined with fluorescence microscopy. Graphs represent mean percentages of apoptotic osteocytes ± SE. (** = $p< 0.001$, compared to H_2O_2 treatment, †= $p<0.01$ compared to control). Control cultures represent untreated cultures. Vehicle cultures for ICI 182 780 at 10^{-6}M were similar to control in percentages of apoptotic osteocytes (5.23 ± 0.2 % S.E., $p>0.05$).

4.3.6 17β-estradiol and 17α-estradiol prevent H_2O_2-induced apoptosis in the ER-negative HeLa cells

To further investigate the potential contribution of the estrogen receptor in the anti-apoptotic effects of 17β-estradiol against H_2O_2 induced death, the ER-negative HeLa cervical epithelial cell line was employed (Rago et al. 2004; Carpino et al. 2004). Administration of H_2O_2 induced apoptosis in HeLa cells in a dose dependent manner between 0.08mM and 0.6mM over a 2 hour incubation period (**Figure 4.8**) while the dose of 0.3mM H_2O_2, that was used to characterise oxidative stress in MLO-Y4 osteocytes, induced comparable levels of apoptosis in HeLa cells and was therefore used in subsequent experiments. Mean percentages of HeLa cells displaying apoptotic morphology were significantly increased compared to the control following treatment with 17β-estradiol at 10^{-6}M ($p<0.0001$), whereas concentrations of 10^{-7}M and 10^{-8}M did not increase the levels of apoptosis above control levels ($p>0.05$) (**Figure 4.9A**). Pre-treatment of HeLa cells with 17β-estradiol for 1 hour at doses of 10^{-6}M, 10^{-7}M and 10^{-8}M all significantly reduced the percentage of H_2O_2 induced apoptosis ($p<0.05$ for 10^{-6}M and 10^{-7}M and $p< 0.001$ for 10^{-8}M), (**Figure 4.9A**). In addition, administration of 17α-estradiol to HeLa cells for 1 hour at concentrations of 10^{-6} M to 10^{-8} M resulted in a significant reduction in the percentage of osteocytes undergoing apoptosis, when compared to H_2O_2 treatment, at all doses used (**Figure 4.9B**). The dose of 10^{-8} M for both 17β-estradiol and 17α-estradiol was used in subsequent treatments since it significantly reduced H_2O_2-induced apoptosis without demonstrating a pro-apoptotic effect on HeLa cells when administered alone in culture.

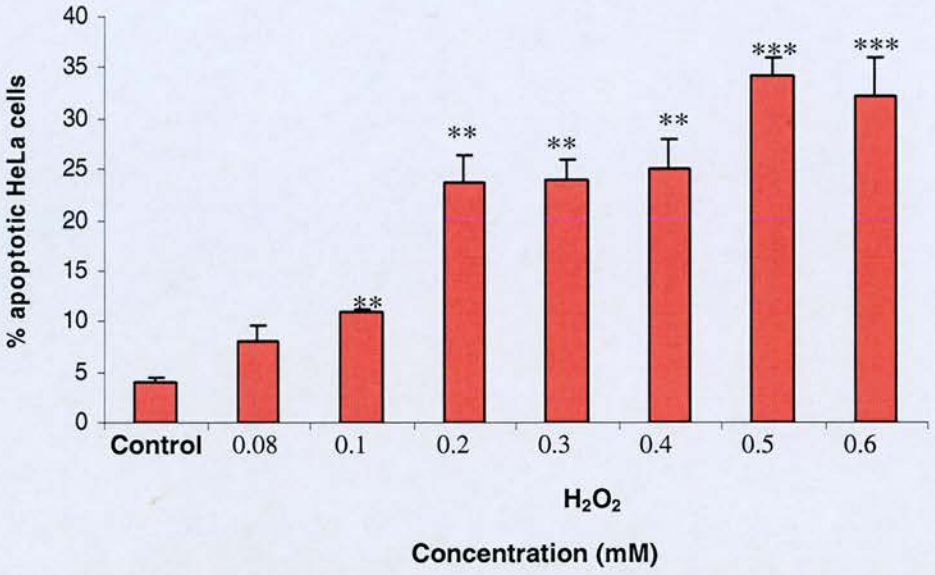
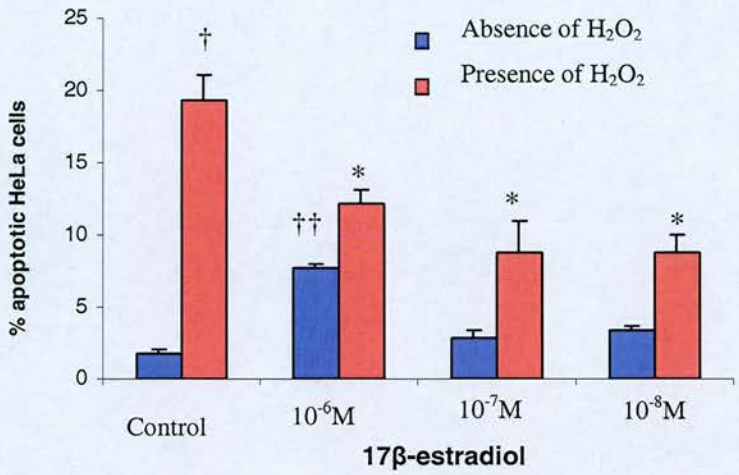


Figure 4.8. H_2O_2 induces apoptosis in HeLa cells in a concentration-dependent manner. HeLa cells were incubated with H_2O_2 at 0.08-0.6 mM for 2 hours. Cell were stained for apoptosis determination with DAPI and examined by fluorescent microscopy. Graphs represent mean percentages of apoptotic HeLa cells ± SE. (*** =p<0.0001, ** = p< 0.001, compared to control). Control cultures represent untreated cultures.

A.



B.

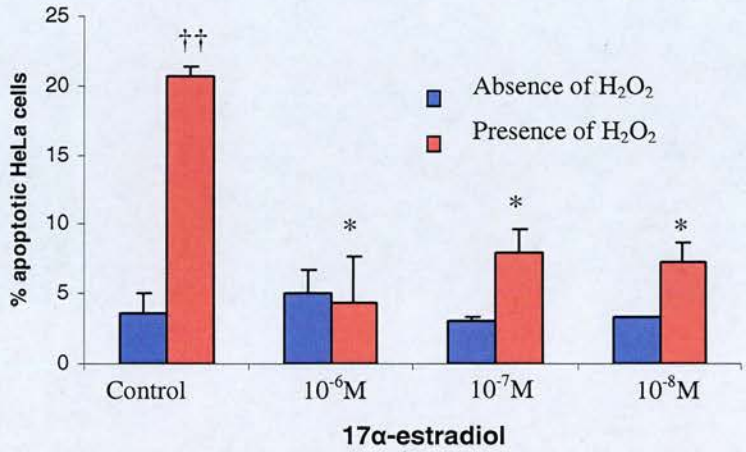


Figure 4.9. 17β-estradiol and 17α-estradiol prevent HeLa cell apoptosis induced by H_2O_2. HeLa cells were incubated with **A.** 17β-estradiol and **B.** 17α-estradiol at 10^{-6} M, 10^{-7} M and 10^{-8} M for 1 hour prior to H_2O_2 treatment for a further 2 hours. Cells were stained with DAPI and examined with fluorescence microscopy Graphs represent mean percentages of apoptotic HeLa cells ± SE. (* = $p< 0.05$ compared to H_2O_2 treatment, ††= $p<0.001$, compared to control). Control cultures represent untreated cultures. Vehicle cultures for 17β-estradiol, 17α-estradiol at 10^{-6}M were similar to control in percentages of apoptotic cells (7.85 ± 0.917 % S.E., $p>0.05$; 4.74 ± 0.322 % S.E., $p>0.05$).

4.3.7 17β-estradiol prevents H_2O_2-induced apoptosis of the ER-negative HEK 293 cells

To further investigate the potential contribution of the estrogen receptor in the anti-apoptotic effects of 17β-estradiol against H_2O_2 induced death, the HEK 293 cell line that lacks the ER was employed (Kahlert et al. 2000, Thomas et al. 2005). The dose of 0.3mM H_2O_2, which was previously chosen in order to characterise oxidative stress in MLO-Y4 osteocytes and HeLa cells, was also employed in the HEK 293 cell line and was found to be within the dose range previously shown to induce oxidative stress-induced apoptosis in HEK 293 cells (Nomura et al. 2006). Pre-treatment of HeLa cells with 17β-estradiol for 1 hour at doses of 10^{-6}M, 10^{-7}M and 10^{-8}M all significantly reduced the percentage of H_2O_2 induced apoptosis ($p<0.05$ for 10^{-6}M and 10^{-7}M and $p< 0.001$ for 10^{-8}M), (**Figure 4.10**).

4.3.8 Inhibition of MAP kinase signalling does not block the protective effect of 17β-estradiol on MLO-Y4 osteocytes.

Previous studies have implicated the ERK ½ pathway in the protective effects of 17β-estradiol against dexamethasone, TNF-α, etoposide in osteocytes (Kousteni et al. 2001, 2003). In order to determine whether the saving effect of 17β-estradiol against H_2O_2 on MLO-Y4 osteocytes was mediated via activation of MAP kinase signalling pathways, the MEK1/2 inhibitor UO126 was used. Pre-treatment of MLO-Y4 with UO126 at the concentration of 20μM previously shown to inhibit ERK phosphorylation (Favata et al. 1998, Kogianni et al. 2004) prior to the addition of 17β-estradiol did not inhibit the anti-apoptotic effects of 17β-estradiol against the H_2O_2 treatment ($p=0.01$, compared to H_2O_2 treatment) (**Figure 4.11**).

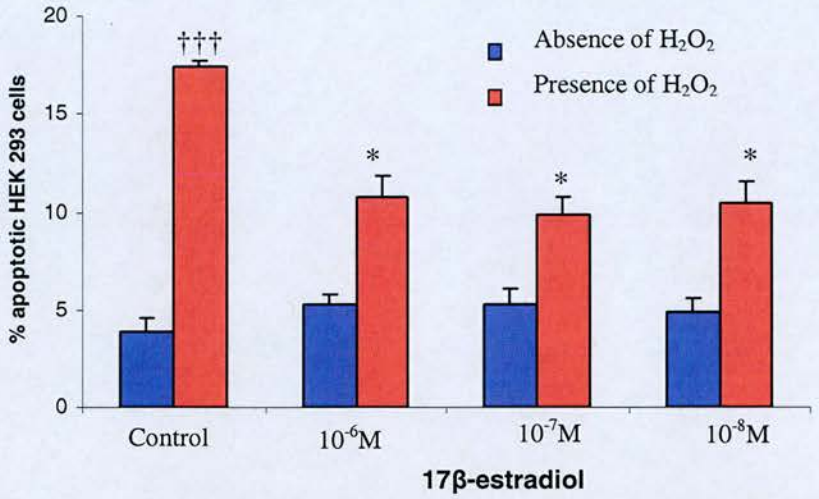


Figure 4.10. 17β-estradiol prevents HEK 293 cell apoptosis induced by H_2O_2. HEK 293 cells were incubated with 17β-estradiol at 10^{-6} M, 10^{-7} M and 10^{-8} M for 1 hour prior to H_2O_2 treatment for a further 2 hours. Cells were stained with DAPI and examined with fluorescence microscopy Graphs represent mean percentages of apoptotic HEK 293 cells ± SE. (* = $p< 0.05$ compared to H_2O_2 treatment, ††† = $p<0.0001$, compared to control). Control cultures represent untreated cultures. Vehicle cultures for 17β-estradiol at 10^{-6}M were similar to control in percentages of apoptotic HEK 293 cells (4.4 ± 0.6 % S.E., $p>0.05$).

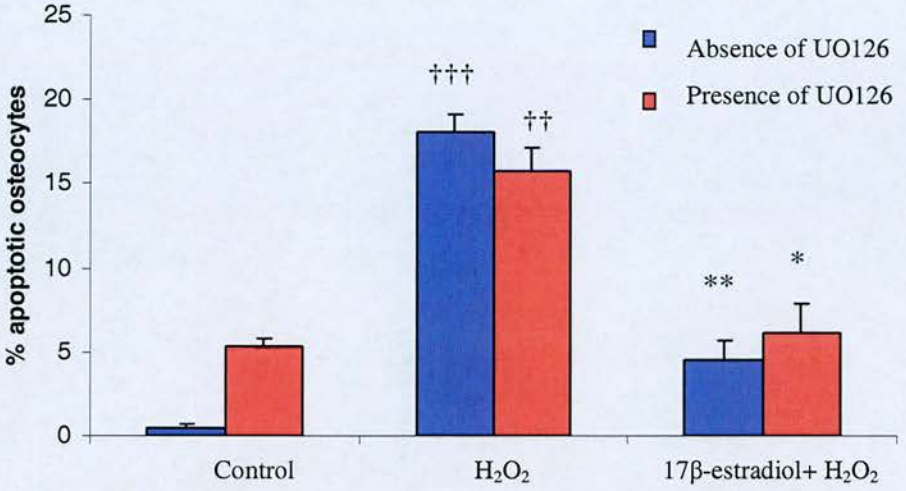


Figure 4.11. The MEK ½ inhibitor U0126 does not inhibit the anti-apoptotic effects of 17β-estradiol. MLO-Y4 osteocytes were incubated with UO126 for 30 minutes at 20 μM prior to the addition of 17β-estradiol for 1 hour in the presence and absence of H_2O_2 at 0.3mM for a further 2 hours. UO126 did not prevent MLO-Y4 osteocyte apoptosis induced by H_2O_2 treatment and did not block the protective effect of 17β-estradiol on MLO-Y4 osteocytes. Cells were stained with DAPI and examined by fluorescence microscopy. Graphs represent mean percentages of apoptotic osteocytes ± SE. (** = $p< 0.001$, * = $p< 0.05$, compared to H_2O_2 treatment, †††= $p<0.0001$, †† =$p<0.001$ compared to control). Control cultures represent untreated cultures and are similar to vehicle (DMSO) cultures in percentages of apoptotic osteocytes.

4.3.9 Prevention of H_2O_2-induced apoptosis in MLO-Y4 osteocytes is associated with the presence of the C3-OH group in the structures of the estrogenic compounds

Comparison of the structures of all the estrogenic compounds that exerted protective effects against H_2O_2-induced apoptosis pointed out that 17β-estradiol, 17α-estradiol and ICI 182 780 all had in common a hydroxyl group (-OH) at the C3 position of their phenolic A ring (**Figure 4.12**). Based on these observations, compounds that did not contain this moiety in their structure, such as mestranol (CH_3O-methyl group instead of the C3-OH group) and quinestrol (cyclopentyl group instead of the C3-OH group) were further utilised against H_2O_2-induced apoptotic cell death.

Dose response studies were used to identify the concentrations of mestranol and quinestrol that did not induce osteocyte apoptotic death when administered alone in culture. Estimation of mean percentages of osteocytes displaying apoptotic morphology showed that mestranol and quinestrol at 10^{-5}M induced significant apoptosis compared to control ($p=0.008$, $p=0.05$, respectively) whereas lower doses such as 10^{-8} M and 10^{-10} M were not different compared to the control levels ($p>0.05$) (**Figure 4.13A, 4.13B**). Mestranol and quinestrol were then used to treat osteocytes prior to addition of H_2O_2 to cultures. Pre-incubation of osteocytes with mestranol or quinestrol, which do not contain the –OH group in their structure, for 1 hour failed to inhibit H_2O_2–induced apoptosis ($p>0.05$), (**Figure 4.13A, 4.13B, 4.14**).

To further determine the dependency of the saving effects of estrogenic compounds against oxidant attack on the presence of the –OH group, 17α-ethinylestradiol was used against H_2O_2-induced apoptosis in MLO-Y4 osteocytes. 17α-ethinylestradiol is structurally similar to mestranol and quinestrol, but with the -methyl and -pentyl groups substituted by C3-OH, respectively (**Figure 4.12**). Estimation of mean percentages of osteocytes displaying apoptotic morphology showed that 17α-ethinylestradiol used at 10^{-5}M induced significant apoptosis compared to control ($p=0.01$) whereas lower doses such as 10^{-8} M and 10^{-10} M were not different compared to the control levels ($p>0.05$)

(**Figure 4.13C**). In contrast to the effects observed with mestranol and quinestrol (**Figure 4.13A, 4.13B**), pre-treatment of osteocytes for 1 hour with 17α-ethinylestradiol prevented pro-apoptotic stimuli induced by H_2O_2 at all concentrations used (**Figure 4.13C, 4.14**).

4.3.10 Prevention of H_2O_2-induced apoptosis in HeLa cells is associated with the presence of the C3-OH group in the structures of the estrogenic compounds

To characterise the contribution of the hydroxyl group present in the estrogenic compounds in the prevention of the oxidative stress in HeLa cells, cultures were pre-treated with 17α-ethinylestradiol, mestranol and quinestrol at doses 10^{-5}, 10^{-8} and 10^{-10}M for 1 hour in the presence or absence of H_2O_2. Estimation of mean percentages of HeLa cells displaying apoptotic morphology showed that 17α-ethinylestradiol prevented H_2O_2 -induced apoptosis (**Figure 4.15A**), whereas neither mestranol nor quinestrol demonstrated similar protective effects at any of the concentrations used (**Figure 4.15B and 4.15C**).

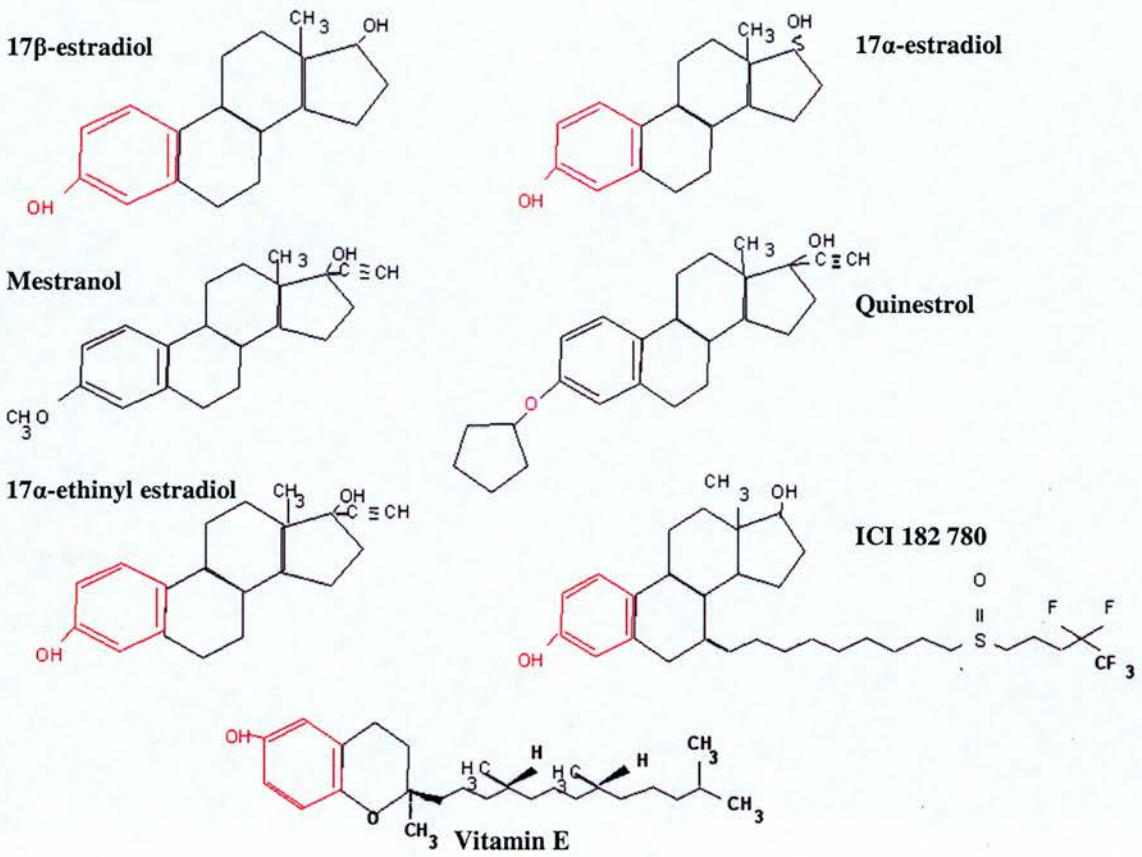


Figure 4.12. Chemical structures of 17β-estradiol and estrogen-related compounds. 17β-estradiol and all related compounds employed in this study, apart from mestranol and quinestrol, are characterized by the presence of a hydroxyl group (-OH group, red) attached to carbon 3 position of their phenolic A ring.

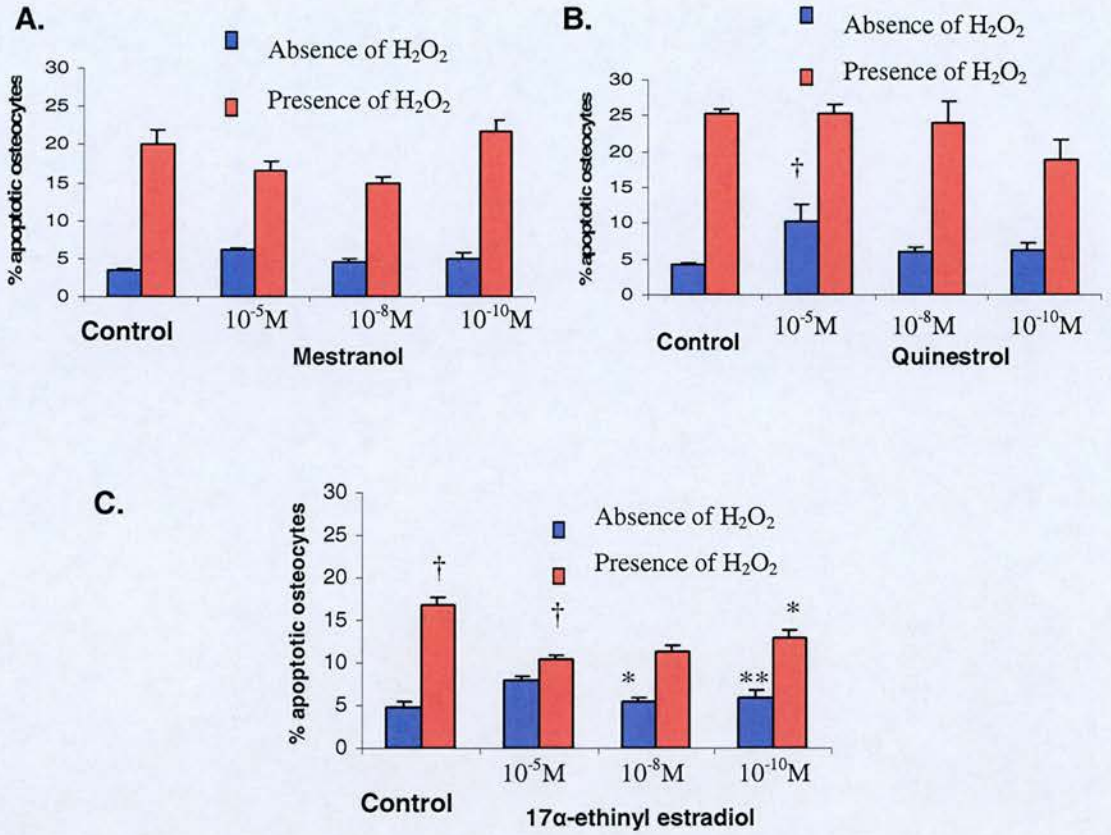


Figure 4.13. Protection against oxidant attack in osteocytes by estrogenic compounds was dependent on the presence of the -OH group. Osteocytes were pre-incubated with **A.** mestranol, **B**. quinestrol and **C**. 17α ethinylestradiol at 10^{-5} to 10^{-10} M for 1 hour followed by addition of H_2O_2 for a further 2 hours. Apoptosis of osteocytes induced by H_2O_2 was not reduced in the presence of **A.** mestranol or **B.** quinestrol. **C.** 17α ethinylestradiol prevented osteocyte apoptosis induced by H_2O_2 treatment at all concentrations used. Cells were stained with DAPI and examined with fluorescence microscopy. Graphs represent mean percentages of apoptotic osteocytes ± SE. (** = $p< 0.001$, * = $p< 0.05$ compared to H_2O_2 treatment, †= $p<0.05$ compared to control). Control cultures represent untreated cultures. Vehicle cultures for mestranol, quinestrol and 17α ethinylestradiol at 10^{-5}M were similar to control in percentages of apoptotic osteocytes (6.9 ± 1.42 % S.E., $p>0.05$; 10.54 ± 1.63 % S.E., $p>0.05$, 7.11 ± 0.22 % S.E., $p>0.05$ respectively).

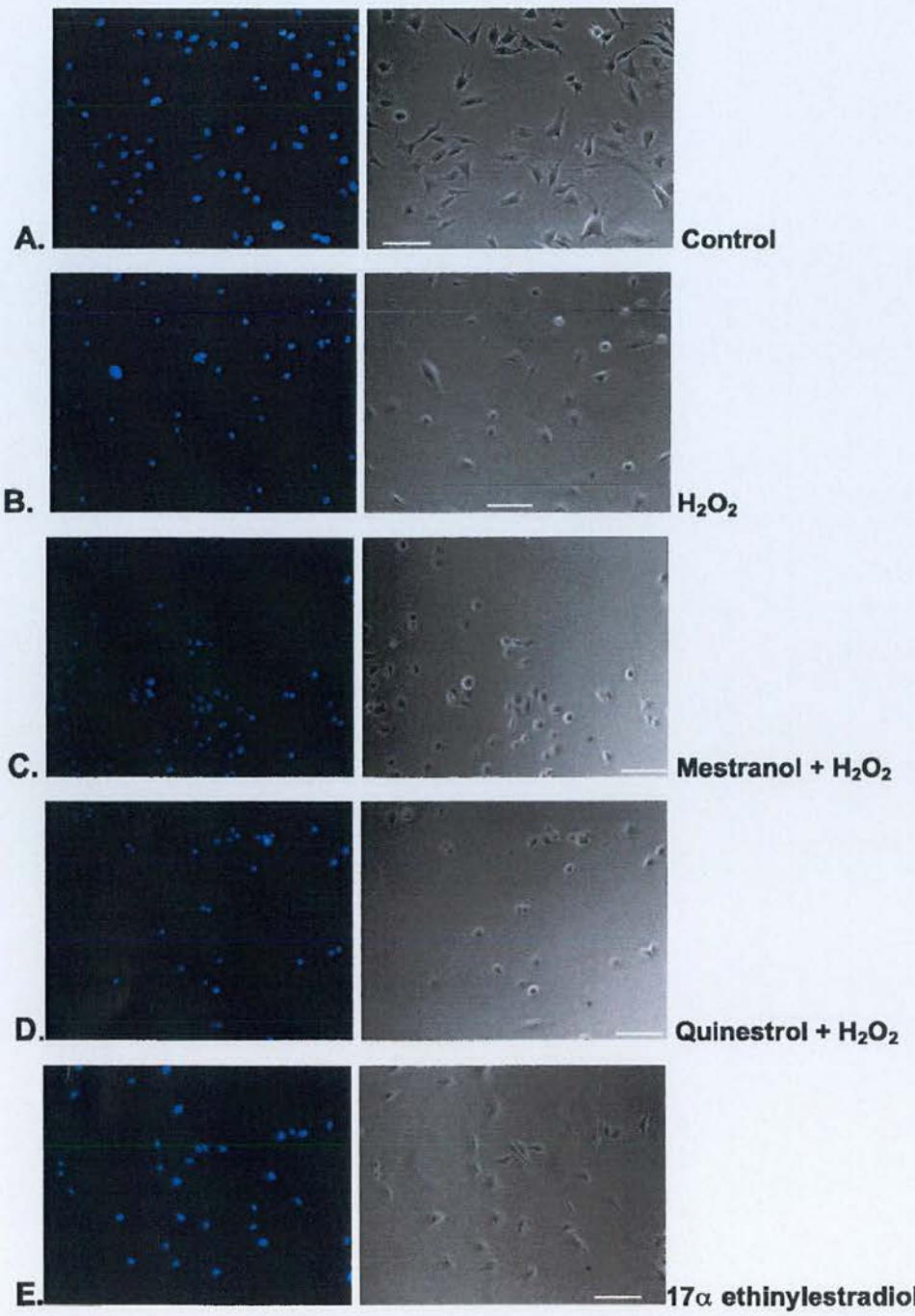


Figure 4.14. The presence of the –OH group determines the prevention of H_2O_2-induced apoptosis in MLO-Y4 osteocytes. Representative images of MLO-Y4 osteocytes treated with either mestranol, quinestrol or 17α ethinyl estradiol for 1 hour followed by H_2O_2 treatment for a further two hours and stained with DAPI stain. In contrast to mestranol (**C**) or quinestrol (**D**), 17α ethinylestradiol (**E**) reduced H_2O_2-induced apoptosis in osteocyte cultures (second panel). Bar = 10 μm.

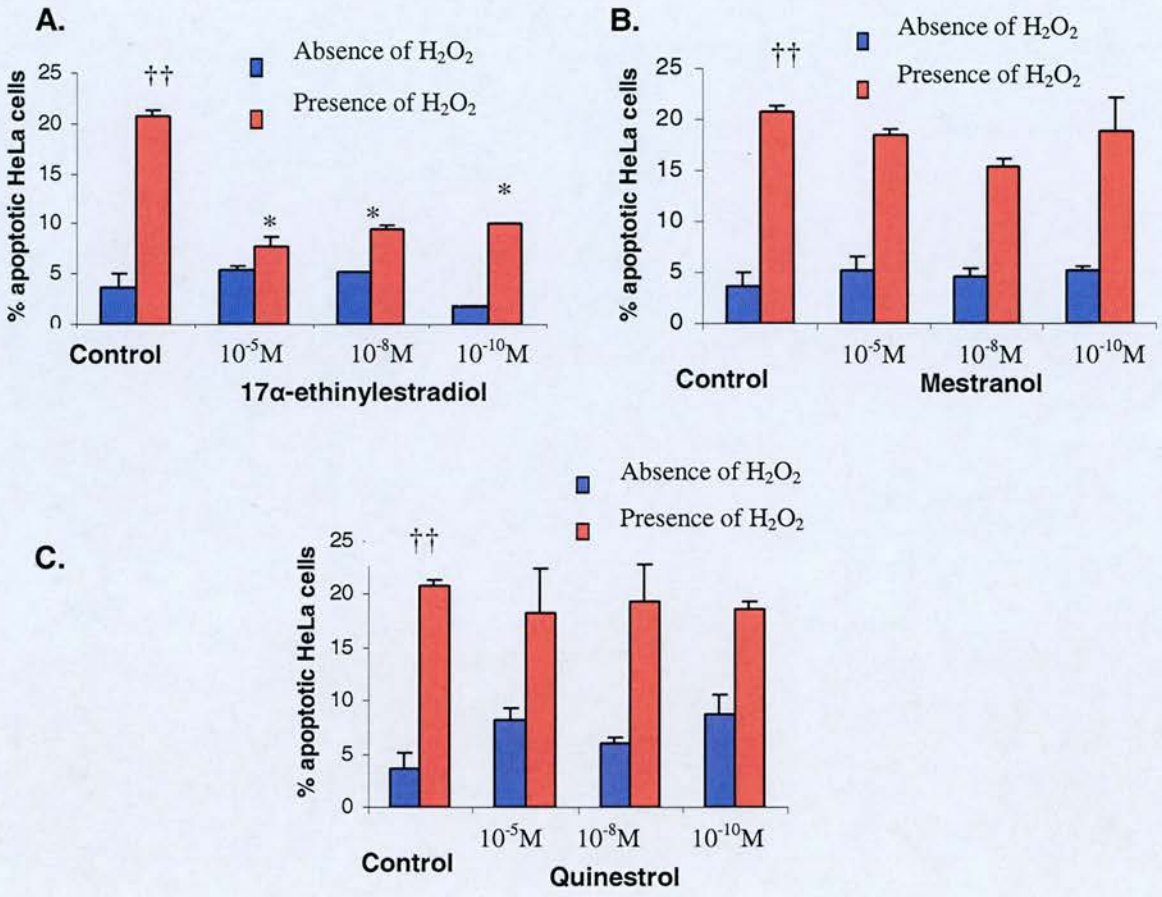


Figure 4.15. Protection against oxidant attack by estrogenic compounds in HeLa cells was associated with the presence of the -OH group. HeLa cells were pre-incubated with **A**. 17α-ethinylestradiol, **B.** Mestranol and **C.** Quinestrol at 10^{-5} M, 10^{-8} M and 10^{-10} M for 1 hour followed by addition of H_2O_2 treatment for 2 hours. **A.** 17α-ethinylestradiol but not **B.** mestranol or **C.** quinestrol prevented H_2O_2 induced apoptosis of HeLa cells at all concentrations used. Cells were stained with DAPI and examined with fluorescence microscopy. Graphs represent mean percentages of apoptotic HeLa cells ± SE. (* =$p<0.05$ compared to H_2O_2 treatment; ††= $p<0.001$ compared to control). Control cultures represent untreated cultures. Vehicle cultures for 17α-ethinylestradiol, mestranol and quinestrol at 10^{-5}M were similar to control in percentages of apoptotic HeLa cells (5.86 ± 0.72 % S.E., $p>0.05$; 4.69 ± 0.6 % S.E., $p>0.05$; 8.48 ± 0.69 % S.E., $p>0.05$, respectively).

4.3.11 Vitamin E prevents H_2O_2-induced apoptosis in MLO-Y4 osteocytes in a dose dependent manner

The anti-oxidant Vitamin E was used to investigate the potential cell protective activity of a known antioxidant compound in this model system. Mean percentages of osteocytes displaying apoptotic morphology were not statistically different to the control following treatment with vitamin E alone at concentrations of 10^{-4}M, 10^{-6}M and 10^{-8}M ($p<0.05$) (**Figure 4.16**). Vitamin E was then used to treat osteocytes prior to addition of H_2O_2 to cultures. Estimation of apoptotic osteocytes showed that pre-incubation of cells for 1 hour with vitamin E at all concentrations used significantly decreased the pro-apoptotic effect of H_2O_2, following 2 hours incubation in osteocyte cultures (**Figure 4.16**). Since the dose used for 17β-estradiol and all the above estrogen-related molecules employed in this study was the lowest effective concentration of 10^{-8} M, the same dose for vitamin E was used in subsequent treatments.

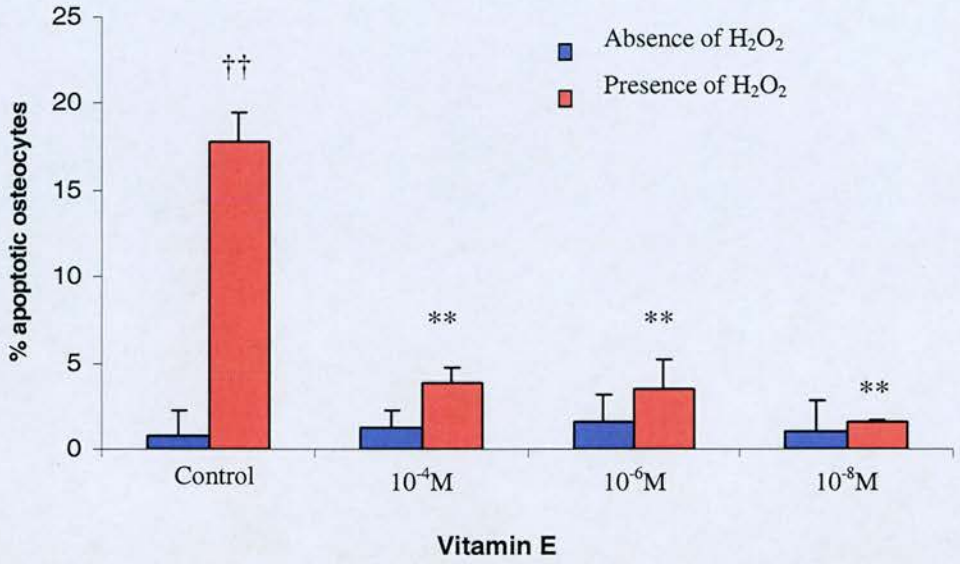


Figure 4.16. Vitamin E prevents MLO-Y4 osteocyte apoptosis induced by H_2O_2 in a concentration dependent manner. Osteocytes were incubated with vitamin E at 10^{-4} M, 10^{-6} M and 10^{-8} M for 1 hour prior to H_2O_2 treatment for a further 2 hours. Vitamin E reduced the mean percentages of apoptotic osteocytes to levels similar to control without itself causing any apoptosis. Cells were stained with DAPI and examined with fluorescence microscopy. Graphs represent mean percentages of apoptotic osteocytes ± SE. (** = $p< 0.001$, compared to H_2O_2 treatment, †† = $p<0.001$ compared to control). Control cultures represent untreated cultures.

4.3.12 17β-estradiol and estrogenic compounds that contain the –OH group reduced the H_2O_2-induced ROS production in MLO-Y4 osteocytes

The ability of H_2O_2 to generate reactive oxygen species in MLO-Y4 was investigated using 2', 7'- dichlorodihydrofluorescein-diacetate (H_2DCF-DA), as described in § 4.2.7. The proportion of ROS positive osteocytes was significantly increased within 2 hours of treatment of osteocytes with H_2O_2 ($p<0.0001$) (**Figure 4.17A, 4.17B, 4.18**). Pre-treatment of osteocytes with compounds that contain the –OH group at C3 position of their phenol A ring such as 17β-estradiol, 17α-estradiol, ICI 182 780, and 17α ethinylestradiol, used at a concentration of 10^{-8}M, significantly reduced the proportion of ROS positive cells following treatment with H_2O_2 to levels similar to those seen in control cultures, as did the potent anti-oxidant Vitamin E (10^{-8}M) (**Figure 4.17A**). In contrast to this observation, pre-incubation of osteocytes with mestranol and quinestrol at 10^{-8}M concentration, failed to reduce the number of ROS positive cells (**Figure 4.17B**).

4.3.13 17β-estradiol and estrogenic compounds that contain the –OH group reduced the H_2O_2-induced ROS production in HeLa cells

The proportion of ROS positive cells was significantly increased within 2 hours of treatment of HeLa cells with H_2O_2 ($p=0.0001$), (**Figure 4.19A and 4.19B**). Pre-treatment of HeLa cells with 17β-estradiol, 17α-estradiol, ICI 182 780 and 17α ethinylestradiol at a concentration of 10^{-8}M, significantly reduced the proportion of ROS positive HeLa cells induced by H_2O_2 treatment ($p=0.004$ for 17β-estradiol and $p<0.05$ for 17α-estradiol, ICI 182 780,17α ethinyl estradiol) (**Figure 4.19A**). In contrast to this observation, pre-incubation of HeLa cells with either mestranol or quinestrol, at a concentration of 10^{-8} M, did not reduce the number of ROS positive cells (**Figure 4.19B**).

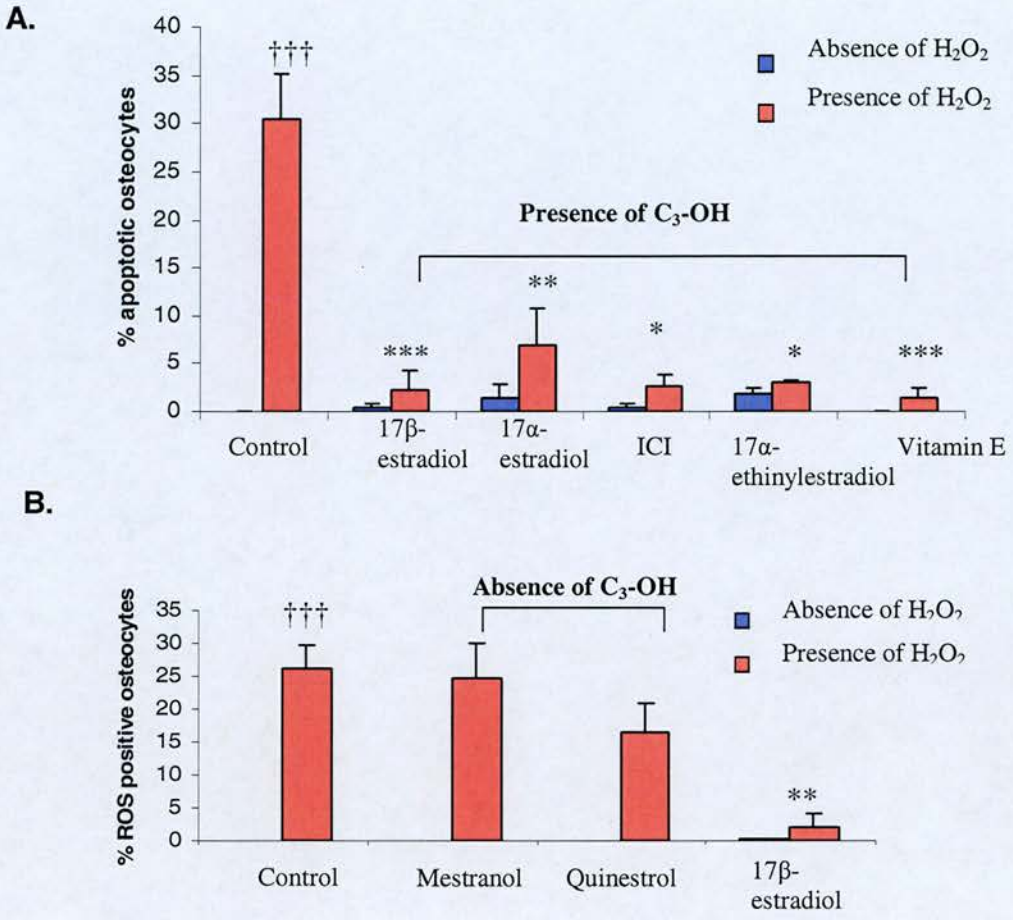


Figure 4.17. Compounds which display the C3-OH moiety in their structure prevent H_2O_2–induced ROS production in osteocytes. MLO-Y4 osteocytes were incubated with H_2DCF-DA for 30 min and then pre-treated with **A.** 17β-estradiol or 17α-estradiol or ICI 182,780 or 17α-ethinylestradiol or Vitamin E for 1 hour prior to addition of H_2O_2 for a further 2 hours. The percentage of ROS positive cells induced by H_2O_2 treatment was reduced in the presence of any of these compounds to levels similar to control. **B.** Pre-treatment of osteocytes with mestranol or quinestrol, which lack the C3-OH group in their structure did not reduce the percentage of ROS positive cells induced by H_2O_2. Cells were examined with fluorescence microscopy. Graphs represent mean percentages of ROS positive osteocytes ± SE. (*** = $p<0.0001$, ** = $p< 0.001$, * = $p<0.05$ compared to H_2O_2 treatment, ††† = $p<0.001$ compared to the untreated control). Control cultures represent untreated cultures.

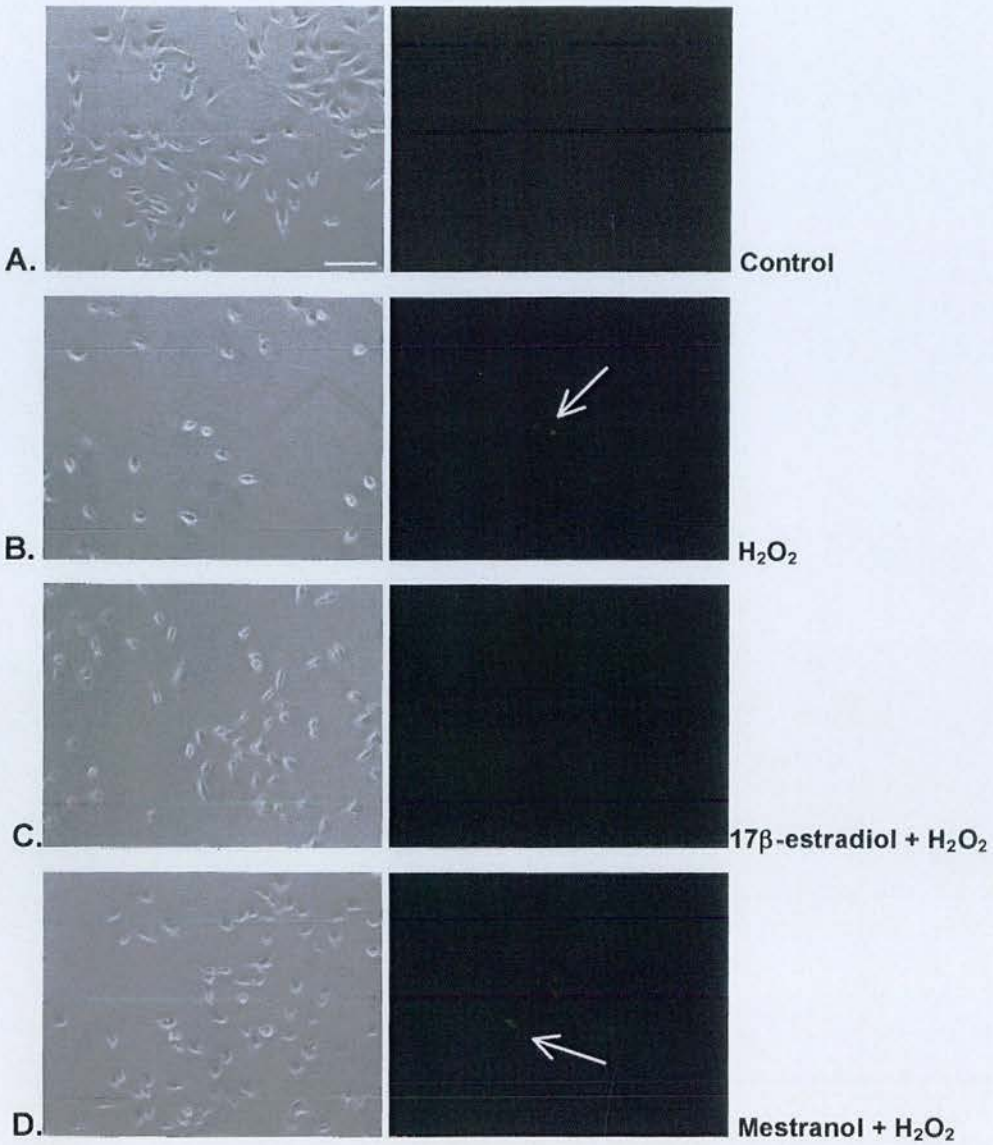


Figure 4.18. Estrogenic compounds that contain the C3-OH moiety in their chemical structures reduced the H_2O_2-induced ROS production in MLO-Y4 osteocytes. MLO-Y4 osteocytes were incubated with H_2DCF-DA for 30 min and then pre-treated with 17β-estradiol and estrogenic compounds for 1 hour prior to H_2O_2 treatment for a further two hours. **A.** control cultures, **B.** H_2O_2-treated cultures, **C.** cultures treated with 17β-estradiol in the presence of H_2O_2, **D.** cultures treated with mestranol in the presence of H_2O_2. White arrows indicate ROS-positive (FITC) osteocytes. Bar = 10μm.

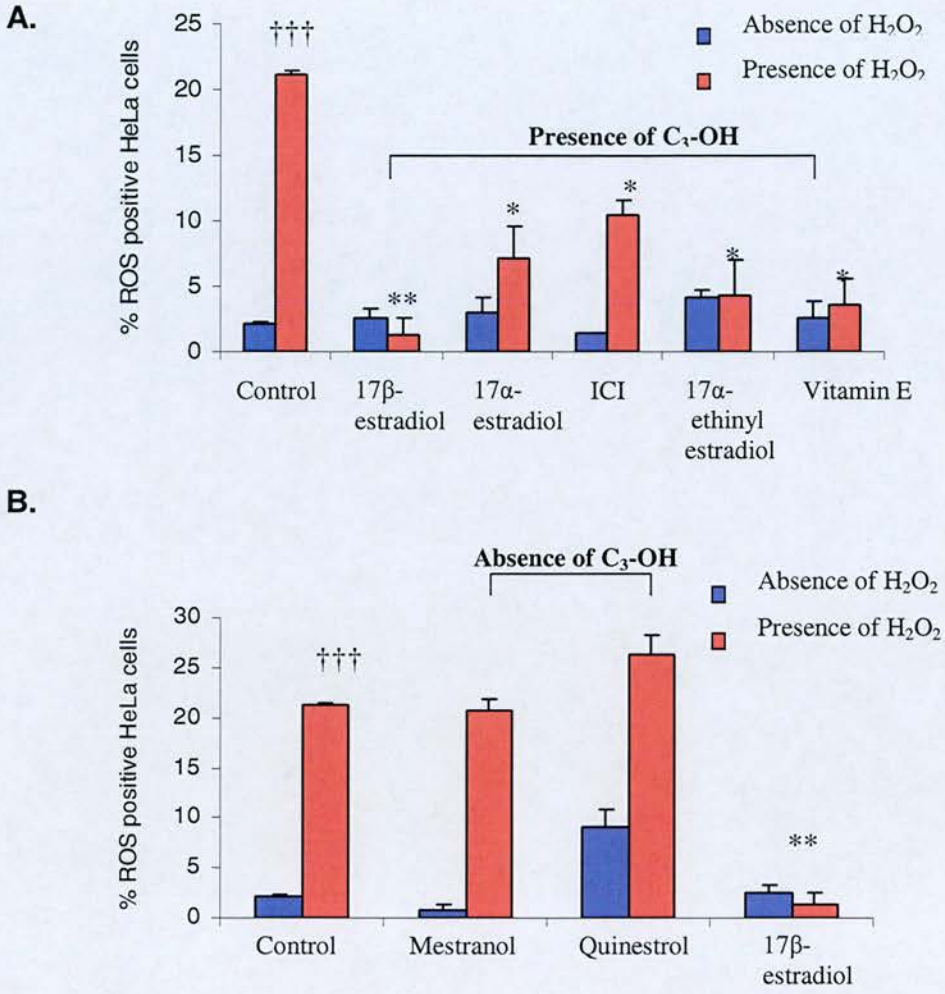


Figure 4.19. Compounds containing the C3-OH moiety in their structure prevent H_2O_2–induced generation of ROS in HeLa cells. HeLa cells were incubated with H_2DCFH-DA for 30 min and then pre-treated with **A.** 17β-estradiol or 17α-estradiol or ICI 182 780 or 17α ethinylestradiol or vitamin E. The percentage of ROS positive cells induced by H_2O_2 treatment was reduced in the presence of any of these compounds to levels similar to control. **B.** Pre-treatment of osteocytes with mestranol or quinestrol, which lack the C3-OH group in their structure did not reduce the percentage of ROS positive cells induced by H_2O_2. Cells were examined with fluorescence microscopy. Graphs represent mean percentages of ROS positive HeLa cells ± SE. (** = $p< 0.001$, * = $p<0.05$, compared to H_2O_2 treatment, †† = $p<0.001$ compared to control). Control cultures represent untreated cultures.

4.3.14 17β-estradiol that contains the –OH group reduced the H_2O_2-induced ROS production in HEK 293 cells

The proportion of ROS positive cells was significantly increased within 2 hours of treatment of HEK 293 cells with H_2O_2 (p=0.04), (**Figure 4.20**). Pre-treatment of HEK 293 cells with 17β-estradiol or vitamin E at a concentration of 10^{-8}M, significantly reduced the proportion of ROS positive HEK 293 cells induced by H_2O_2 treatment (p=0.04 and p=0.01, respectively) (**Figure 4.20**).

4.3.15 17β-estradiol prevents H_2O_2-induced osteocyte apoptosis in murine calvariae *ex vivo*.

In order to demonstrate protection of osteocytes with 17β-estradiol against H_2O_2-induced oxidative stress ex vivo, calvariae derived from 12 Balb/c mice were incubated with 17β-estradiol at 10^{-8}M for 1 hour in the presence or absence of H_2O_2 for 24 hours. Estimation of osteocyte apoptosis indicated that H_2O_2 induced bone resident osteocytes to undergo apoptosis (p=0.02) (**Figures 4.21, 4.22**). Estimation of mean percentages of osteocytes undergoing apoptosis indicated that 17β-estradiol significantly reduced in H_2O_2-induced osteocyte apoptosis compared to mean percentages of osteocyte apoptosis in the control cultures (p=0.02) (**Figure 4.21**).

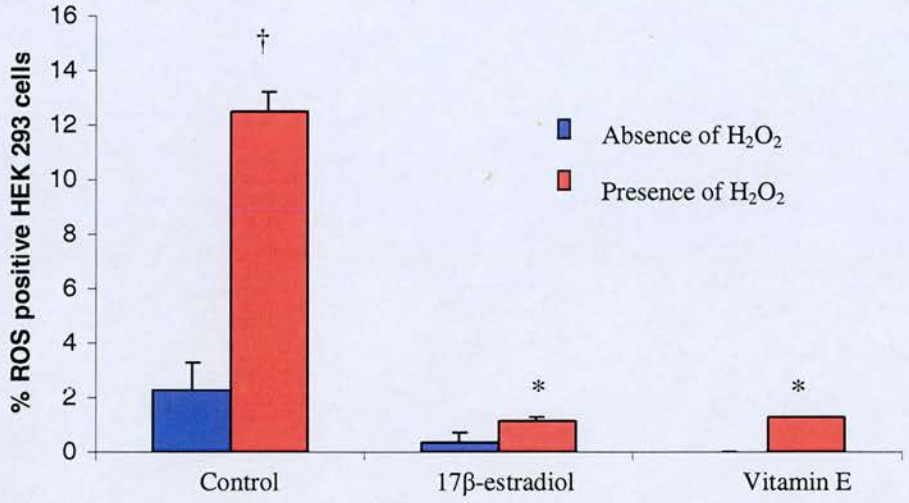


Figure 4.20. 17β-estradiol that displays the C3-OH moiety in its structure prevents H_2O_2–induced generation of ROS in HEK 293 cells. HEK 293 cells were incubated with H_2DCFH-DA for 30 min and then pre-treated with 17β-estradiol, vitamin E for 1 hour prior to addition of H_2O_2 for a further 2 hours. Pre-treatment with 17β-estradiol or vitamin E reduced the percentage of ROS positive cells induced by H_2O_2 treatment to levels similar control. Cells were examined with fluorescence microscopy. Graphs represent mean percentages of ROS positive HEK 293 cells ± SE. (* = $p< 0.05$, compared to H_2O_2 treatment, † = $p<0.05$ compared to control). Control cultures represent untreated cultures.

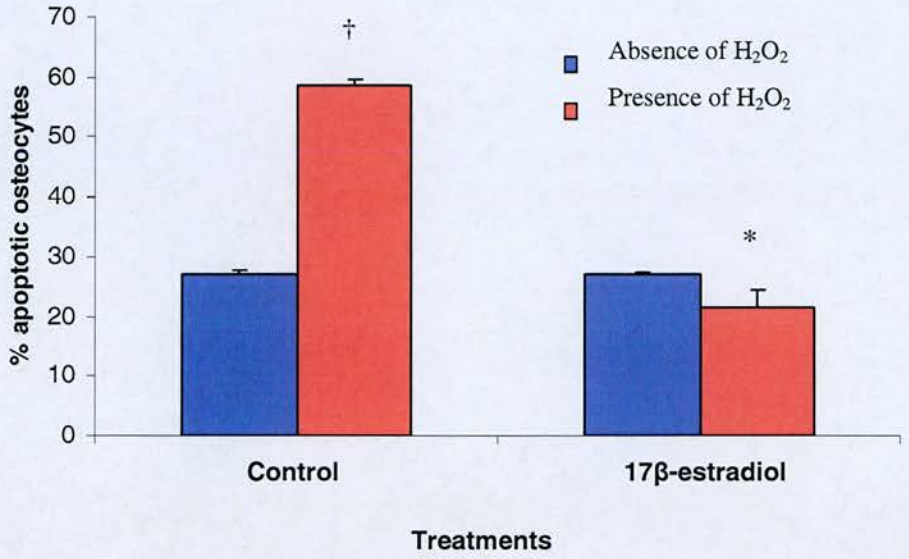


Figure 4.21. 17β-estradiol prevents osteocyte apoptosis induced by H_2O_2 in murine calvariae *ex vivo*. Murine calvaria were incubated with 17β-estradiol at 10^{-8}M for 1 hour in the presence or absence of H_2O_2 for a further 24 hours. Mean percentages of osteocytes undergoing apoptosis in response to H_2O_2 were increased compared to control, while pretreatment of calvariae with 17β-estradiol reduced H_2O_2-induced osteocyte apoptosis to levels similar to control. Osteocytes were stained with DAPI and examined with fluorescence microscopy. Graphs represent mean percentages of apoptotic osteocytes ± SE. (* = $p< 0.05$ compared to H_2O_2 treatment, † = $p<0.05$ compared to control).

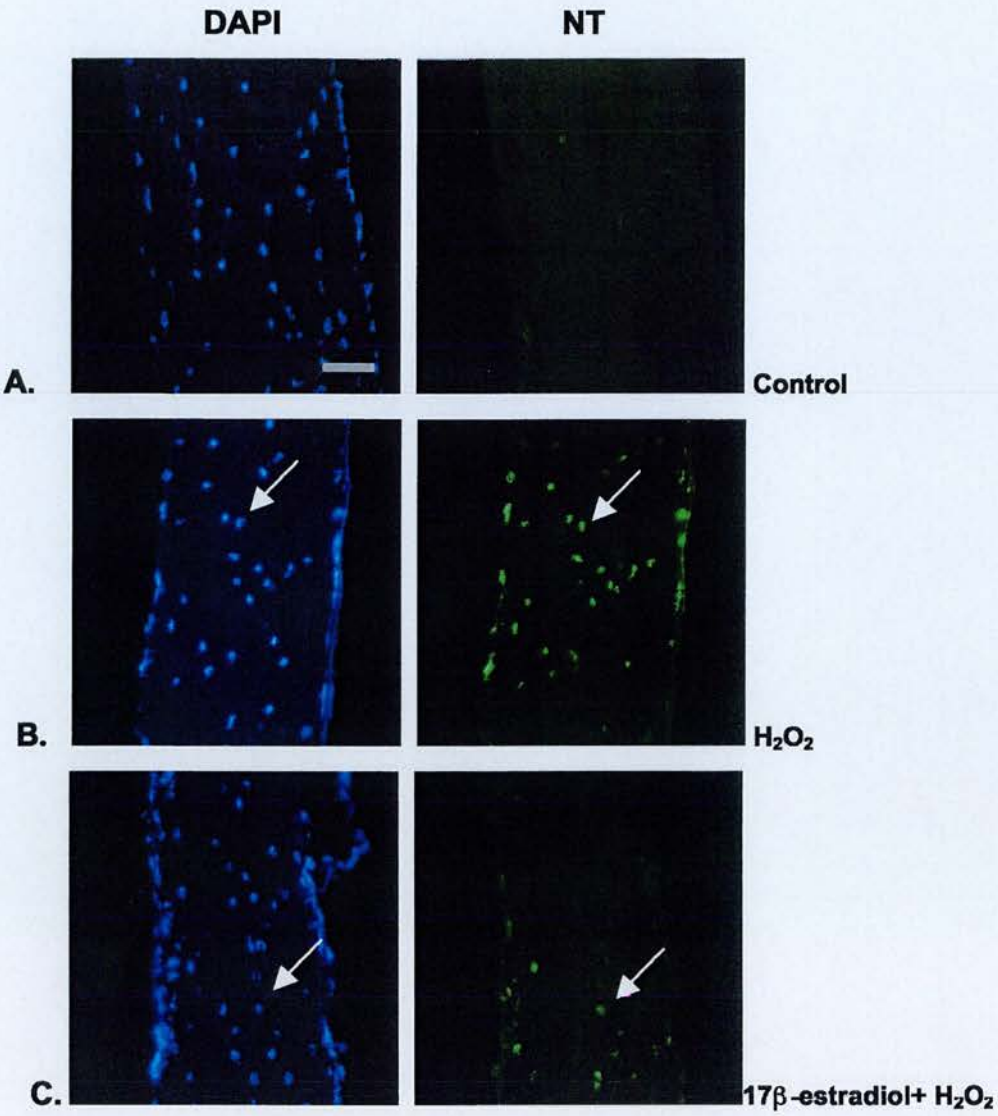


Figure 4.22. 17β-estradiol prevents osteocyte apoptosis induced by H_2O_2 in murine calvaria *ex vivo*. Representative images of murine calvarial osteocytes ex vivo treated with 17β-estradiol for 1 hour followed by H_2O_2 treatment for a further 24 hours and reacted with the nick translation mixture in order to identify apoptotic osteocytes (FITC-green). H_2O_2 increased osteocyte apoptosis (**B**) which was reversed by the presence of 17β-estradiol (**C**). White arrows illustrate osteocytes staining positive for DNA fragmentation (FITC-green). (x20). Bar = 100 μm.

4.4 Discussion

This study has shown that exposure of MLO-Y4 osteocytes to H_2O_2, one of the major reactive oxygen species, resulted in oxidative stress-induced apoptotic cell death characterised by membrane blebbing and condensed chromatin at concentrations between 0.08mM and 0.3mM (**Figure 4.1**). These data are in agreement with the work of Kikuyama et al in which similar concentrations of H_2O_2 were used to induce apoptosis in chick-derived primary osteocytes (Kikuyama et al. 2002). At higher concentrations of H_2O_2, above 0.4mM and up to 0.6mM, osteocytes were shown to die via necrosis, as has previously been noted (Kogianni et al. 2004), and therefore were not employed in these experiments.

Several studies have reported that increased intracellular ROS production might play a role in apoptotic cell death possibly by activating certain caspase cascades. For example, H_2O_2 has been shown to trigger apoptosis in primary osteocytes (Kikuyama et al 2002) and T cells (Dumont et al. 1999) possibly by disrupting the mitochondrial membrane potential resulting in cytochrome c release, activation of caspase 9 and subsequent activation of caspase 3 (Dumont et al. 1999). In order to demonstrate the apoptotic nature of the cell death observed in response to H_2O_2, a selective inhibitor of caspase 3/7 based on isatin sulfonamide was employed at concentrations previously shown to inhibit caspases 3 and 7 in osteocytes (Kogianni et al. 2004). Inhibition of caspase 3/7 activity was shown to prevent the H_2O_2-triggered death of MLO-Y4 osteocytes (**Figure 4.3**) indicating that this cell death was caspase- dependent and therefore apoptotic.

Pre-treatment of MLO-Y4 osteocytes with 17β-estradiol at 10^{-8}M in the presence of H_2O_2 resulted in an approximate 60% reduction in H_2O_2-induced apoptosis to levels similar to those observed in control cultures (**Figures 4.4, 4.5**). The concentration of 10^{-8}M employed in this study is close to the physiological serum concentration of 17β-estradiol during a typical menstrual cycle in humans (Ganong 1999, Dotsch et al. 2001), and was shown to be non-toxic to osteocyte cultures, in accordance to other studies

(Kousteni et al. 2001, 2003; Gu et al. 2005). Similar protective effects of 17β-estradiol were also demonstrated in murine calvariae exposed to H_2O_2 indicating the ability of 17β-estradiol not only to protect MLO-Y4 osteocytes in culture but also to protect the osteocytic population against oxidant attack in ex vivo cultures (**Figure 4.21, 4.22**).

The concentration of 10^{-8}M employed in this study was shown to be non-toxic to osteocyte cultures, in accordance to other studies (Kousteni et al. 2001, 2003; Gu et al. 2005). These studies have also shown that the use of 17β-estradiol at concentrations lower than the 10^{-8} M, such as 10^{-9}M -10^{-12}M, protected the osteocytes from etoposide (Kousteni *et al* 2001, Kousteni *et al* 2003) and dexamethasone-induced apoptosis (Gu *et al* 2005). However, lower concentrations of 17β-estradiol were not included in this study since it was influenced by the large body of literature describing the concentration of 10^{-8}M as being close to the normal physiological range. In addition, investigation of the effects of lower estrogen doses, closer to the physiological range, against osteocyte apoptosis would have required modifications of the culture conditions such the use of phenol red-free medium or use of charcoal-stripped serum.

In contrast to the effects observed with 17β-estradiol at 10^{-8}M, pre-treatment of MLO-Y4 osteocytes with 17β-estradiol at 10^{-6}M, a concentration that is closer to the pharmacological concentration, appeared to be toxic in culture as it induced osteocyte apoptosis (**Figure 4.4**). Although 17β-estradiol used at 10^{-6}M reduced pro-apoptotic stimuli induced by H_2O_2 in this study (**Figure 4.4**) and by etoposide in other studies (Kousteni et al. 2001), its ability to induce apoptosis when administered alone in osteocytic cultures has not, to the author's knowledge, been demonstrated before. In general, consideration of the effects of the different doses of 17β-estradiol against oxidant-induced osteocyte apoptosis employed in this study might give important insight into the pharmacological properties of 17β-estradiol.

17β-estradiol has previously been reported in the literature to protect osteocytes against apoptosis induced in response to dexamethasone (Kousteni et al. 2001, Gu et al. 2005), etoposide and TNF-a (Kousteni et al. 2001) possibly through a non-genotropic estrogen-

presence of UO126 at concentrations known to block ERK phosphorylation in the MLO-Y4 cell type (Kogianni et al. 2004), (**Figure 4.11**). The failure of UO126 to abolish 17β-estradiol-induced survival of osteocytes might be related to the nature of the death stimulus per se. Studies that have pointed to the involvement of ERK 1/2 pathway in the anti-apoptotic effects of 17β-estradiol have utilised dexamethasone, etoposide or TNF-α as pro-apoptotic stimuli (Kousteni et al. 2001, 2003), whereas in this study death was induced in response to a direct oxidant molecule (H_2O_2).

MLO-Y4 osteocytes have been demonstrated to express both estrogen receptors (ERα and ERβ) (Matikainen and Vaananen 1999). In order to provide evidence for the possible implication of the estrogen receptor in the protective effect of 17β-estradiol against H_2O_2-induced death of MLO-Y4 osteocytes, the widely used pure estrogen receptor antagonist ICI 182 780 was employed. ICI 182 780 is thought to compete with estrogen for binding to the estrogen receptor on which it binds with similar affinity to 17β-estradiol (Wakeling et al. 1991) and disrupts the receptor shuttling process by inhibiting nuclear uptake of the receptor (Dauvois et al. 1993). ICI 182 780 has been successfully shown in other studies to block the protective effects of 17β-estradiol against cell death indicating the possible dependency of these effects on the activation of the classical estrogen receptor (Wilson et al. 2000, Callier et al. 2001, Gu et al. 2005, Kousteni et al. 2001, 2003). In this study, the protection conferred by 17β-estradiol to osteocytes was not reversed by the presence of ICI 182 780 (**Figure 4.7A**) suggesting, at first inspection, lack of the classical estrogen receptor mediation in the protection against oxidative stress. This finding was in accordance with other studies which have also suggested the inability of ICI 182 780 to block the anti-apoptotic effects of 17β-estradiol pointing to the possible employment of the estrogen receptor-independent mechanisms (Behl et al. 1999; Biewenga et al. 2005, Wang et al. 2006).

Interestingly, incubation of osteocytes with ICI 182 780 alone also appeared to prevent osteocyte apoptosis induced by exposure to H_2O_2 and, therefore, the use of ICI 182 780

compound was not sufficient to elucidate lack of estrogen receptor involvement in the anti-apoptotic effect of 17β-estradiol. For this reason, 17α-estradiol and the ER-negative HeLa and HEK 293 cell lines were also employed in this study. 17α-estradiol, which is a stereoisomer of 17β-estradiol (**Figure 4.12**) that acts as a weak estrogen receptor agonist (Huggins et al. 1954, Korenman et al. 1969, Clark et al. 1982, Kneifel et al. 1982, Clark and Markaverich 1983, Lubahn et al. 1985), is thought to be unable to activate the classical ER-mediated transcription (Gorski et al. 1994). Interestingly, both α and β isomers of estradiol were equally effective in protecting osteocytes against oxidant-induced apoptosis when used at the same concentration (**Figures 4.4, 4.6**) possibly indicating that the positive effect of 17β-estradiol on osteocyte survival could be mediated via a mechanism that may not require the classical ER-mediated transcription. These findings are in agreement with other studies which have also shown that 17α-estradiol was equally effective as 17β-estradiol in protecting mouse HT22, rat primary neurons and human SK-N-SH neuronal cell lines from the cytotoxic effects of amyloid β protein (Behl et al. 1997) and serum deprivation, respectively (Green et al. 1997). In addition, the ability of 17β-estradiol to inhibit H_2O_2-induced apoptosis in the HeLa cell line, which is believed to lack estrogen receptors (Rago et al. 2004; Carpino et al. 2004) (**Figure 4.9A**), further supported the possible lack of involvement of the estrogen receptor in the blockade of apoptosis in osteocytes under oxidant attack. However, although HeLa cells were initially thought not to express the ER, it is now believed that some subtypes of the cell line might actually be expressing small amounts of the receptor (Monje and Boland 2002). Based on these controversial reports, another cell line the HEK 293, which has been shown to be ERα and ERβ negative (Kahlert et al. 2000, Thomas et al. 2005), was also employed in this study and showed the same protective effect of 17β-estradiol against H_2O_2-induced apoptosis of HEK 293 cells.

Based on these observations and on the fact that the ER antagonist ICI 182, 780 also prevented H_2O_2-induced apoptosis in osteocytes (**Figure 4.7B**), alternative mechanisms that could explain the anti-apoptotic effects of 17β-estradiol and estrogenic compounds

on osteocytes were investigated in this study. Estrogen has been shown in a number of studies to exert direct antioxidant activity mainly in the neuronal system (Behl et al. 1995, 1997, 2003; Green et al. 1997, Miranda et al. 2000). The prevention of oxidative stress-induced cell death in neurons by estrogen is believed to occur through its direct anti-oxidant activity that is associated with the presence of the hydroxyl (-OH) group at C3 position of the phenolic A ring (Behl et al. 1997, 1999, Green et al. 1997). The presence of the C3-0H group is a common characteristic (a fundamental component of antioxidant activities) of all antioxidant molecules including that of the well known antioxidants such as vitamin E (Behl et al. 1992, Sano et al. 1997). This moiety augments the antioxidant molecule to neutralize (detoxify) the accumulated ROS by donating the proton atom of the phenol group to the unstable radicals in order to terminate the propagation oxidative chain within the mitochondria (Behl et al. 2002). Previous studies have demonstrated that estrogens as well as estrogenic derivatives that contain this C3–OH group in their phenolic A ring protect neurons from oxidant attack in a receptor-independent manner suggesting that the neuro-protective effects of 17β-estradiol are possibly related to free radical scavenging properties (Behl et al. 1995, 1997, Green et al. 1997, Sawada et al. 1998, Mossmann and Behl 1999).

In this regard, consideration of the structure of the molecules employed in this study (**Figure 4.12**) provided possible insight into the anti-apoptotic nature of these compounds in MLO-Y4 osteocytes. 17β-estradiol and 17α-estradiol contain the C3-OH group in their structures and so does the pure estrogen receptor antagonist ICI 182 780. The presence of the C3-OH group in the structure of ICI 182 780 might account for the potential ability of ICI 182 780 to engender anti-oxidant activity, a characteristic that would explain its ability to save osteocytes against oxidant attack in the absence of 17β-estradiol.

To determine the involvement of the C3–OH group in the protection of MLO-Y4 osteocytes from H_2O_2 attack, an estrogen related compound mestranol was utilised against oxidative stress in osteocytes. Mestranol which contains a methyl group (-

CH_3O) in the C3 position of the steroid A ring (**Figure 4.12**), was unable to protect osteocytes from H_2O_2-induced apoptosis (**Figures 4.13A, 4.14C**) while the mestranol's active metabolite 17a-ethinylestradiol, which retains the C3–OH group but is in all other aspects identical to mestranol in structure, was capable of preventing H_20_2-induced osteocyte apoptosis (**Figures 4.13C, 4.14E**). These findings concur with those in neuronal cell types; in which mestranol was employed in a similar context (Behl et al. 1997) indicating that upon introduction of a methyl group in the phenolic A ring of mestranol the antioxidant activity was lost. The inability of mestranol to protect the osteocytic population was further supported by the use of quinestrol (**Figures 4.13B, 4.14D**), another estrogen derivative which contains a cyclopentyl group in the C3 position of the steroid A ring (**Figure 4.12**), further pointing to the importance of the C3-0H group in the saving mechanism of 17β-estradiol against oxidative stress-induced apoptosis in osteocytes. Similar observations were made using the HeLa and the HEK293 cells, further supporting the finding that protective effects against oxidative stress were conferred only by the compounds that contained the OH group in their structures.

Furthermore, the ability of these estrogen related compounds to reduce ROS generation was tested by the use of dichlorofluorescein-diacetate (H_2DCF-DA), one of the most widespread methods used in order to detect ROS. All of the compounds tested in this study that contained the C3-OH group, apart from mestranol and quinestrol, appeared to reduce ROS generation in osteocytes **(Figure 4.17A, 4.17B, 4.18)**, HeLa **(Figure 4.19A, 4.19B)** and HEK 293 cells that lack the ERs **(Figure 4.20)**. These findings possibly support the hypothesis that the anti-apoptotic effects of 17β-estradiol against oxidative stress could be associated with a possible direct free radical scavenging activity of 17β-estradiol and estrogenic compounds that contained the C3-OH group (**Figure 4.12**). Although this method was simple and relatively easy to perform there have been reports questioning the sensitivity of this assay since the use of this probe maybe an indicator of the cellular redox status rather than an estimation of intracellular ROS production. However, the use of the dye combined with fluorescence microscopy allowed the

quantification of the exact number of the cells that produced ROS in culture at the time of investigation rather than an estimation of the total amount of fluorescence produced in each culture as permitted for example by the use of the fluorimeter (Bejma et al. 2000).

Oxidative stress that results from an imbalance between the pro- and anti-oxidant status in a cell leads to increased accumulation of ROS in the aged and has been suggested to contribute to the degeneration of biological functions and the pathogenesis of age-related diseases (Finkel and Holbrook 2000). Production of ROS in bone has been shown to participate in the process of bone resorption (Garrett et al. 1990) and to be involved in osteoclastogenesis both in vitro and in vivo (Lean et al. 2003). Previous evidence has suggested that increased oxidative stress has been associated with reduced BMD possibly playing a role in the etiology of osteoporosis (Basu et al. 2001). Furthermore, *in vivo* studies have demonstrated that estrogen deficiency due to ovariectomy (OVX) results in increased accumulation of oxidants by down regulating the level and the activity of antioxidant enzymes such as glutathione peroxidase 1 in osteoclasts (Lean et al. 2003, 2005). Preliminary data in this group have provided evidence on the presence of antioxidant enzymes such as glutathione peroxidase 1 and catalase in MLO-Y4 osteocytes. However, further experiments are required to determine any possible involvement of these enzymes in the protection of osteocytes against oxidative conditions.

Since results presented in this thesis demonstrate that osteocytes are sensitive to the levels of ROS, it might be important to consider situations under which osteocytes would be exposed to ROS in vivo. Cellular ROS generation in response to hypoxia is known to be part of the signaling response of the contractile process in cardiomyocytes (Duranteau et al. 1998). In bone, osteocyte hypoxia has been shown to occur under disuse conditions suggesting that this forms part of a novel mechanotransduction pathway (Gross et al. 2001). Osteocyte apoptosis is closely associated with regions of osteoclastic resorption leading to the suggestion that signals derived as a result of osteocyte apoptosis may act as a specific target for bone resorption (Noble et al. 2003).

Maintenance of ROS homeostasis might be very important since estrogen depletion represents a double jeopardy to bone. Not only does estrogen loss increase osteocyte apoptosis (Tomkinson et al. 1997, 1998) which in turn may act to target osteoclastic resorption but estrogen depletion causes increased ROS generation through negative feedback on glutathione peroxidase 1 (Lean et al. 2005) resulting in further increased osteoclast activity. Therefore, osteocytes could be exposed to sustained production of ROS as a result of local generation as a result of active resorption, conditions of disuse-induced hypoxia, mechanical load engendered cell signaling, estrogen deficiency conditions or following local inflammatory responses.

It has been shown that estrogen plays an important role in the maintenance of osteocyte viability (in the control of osteocyte apoptosis) but the mechanism of estrogen action on osteocyte apoptosis is still unknown. In conclusion, this study introduces a novel mechanism of estrogen action in the maintenance of the osteocytic population in culture against oxidant-induced apoptosis of osteocytes and points to the importance of understanding all aspects of estrogens activity in bone.

CHAPTER 5

The anti-oxidant effect of raloxifene and LY 117018 in the inhibition of osteocyte apoptosis *in vitro*.

Abstract

This work is the subject of a published manuscript. Data in chapter 4 have suggested that 17β-estradiol and estrogen-like compounds prevented osteocyte apoptosis induced by H_2O_2 possibly by exerting antioxidant properties in vitro. In addition, data in chapter 3 have demonstrated that the LY 117018 SERM is capable of mimicking the protective effects of 17β-estradiol against OVX-induced osteocyte apoptosis in a rat model in vivo. Based on these findings, the hypothesis investigated in this study was that raloxifene and the LY 117018 SERM would protect MLO-Y4 osteocytes from H_2O_2-induced apoptosis by exerting antioxidant properties, as effectively as previously demonstrated for 17β-estradiol and related compounds.

MLO-Y4 osteocyte cultures were incubated with H_2O_2 in the absence or presence of raloxifene and LY 117018. Estimation of apoptotic osteocytes showed that both SERMs were equally effective as 17β-estradiol in preventing H_2O_2-induced osteocyte apoptosis. Importantly, the receptor antagonist ICI 182, 780 did not prevent the anti-apoptotic effects of both SERMs against H_2O_2-induced apoptosis in MLO-Y4 osteocytes. Pre-treatment of the ER-negative HeLa and HEK 293 cells with raloxifene or LY117018 significantly inhibited H_2O_2-induced apoptosis in both these cell types possibly indicating the induction of an estrogen receptor-independent mechanism of action employed by these compounds for the prevention of oxidative stress. The protective effect exerted by raloxifene or LY 117018 on osteocytes appeared to be independent of the ERK1/2 pathway since UO 126 failed to prevent the anti-apoptotic effects of raloxifene or LY 117018 in the presence of H_2O_2. Furthermore, both SERMs significantly reduced the generation of ROS in MLO-Y4 osteocytes, HeLa and HEK 293 cells in a similar manner to 17β-estradiol, suggesting that both raloxifene and LY 117018 possess anti-oxidant activity.

Evidence presented in this chapter has suggested that both raloxifene and LY 117018 are capable of mimicking the protective effects of 17β-estradiol against oxidant-induced apoptotic death of osteocytes by acting as antioxidant molecules.

5.1 Introduction

Selective Estrogen Receptor Modulators (SERMs) are non-steroidal molecules with tissue specific receptor-mediated effects on the various estrogen target tissues (McDonnell et al. 2000, Sandberg et al. 2002). In bone, SERMs are thought to retain the positive effects of estrogen without exerting the adverse effects of estrogen on breast and uterine tissue (Kauffman et al. 1995, Dodge et al. 1997). Furthermore, SERMs have been shown to be effective anti-resorptive agents in vivo and, in particular, raloxifene has been extensively used for the prevention and treatment of postmenopausal osteoporosis (Delmas et al. 1997).

Raloxifene and the raloxifene analogue LY 117018 employed in this study have similar chemical structures and belong to the benzothiophene group characterised by the presence of the benzothiophene ring (**Figure 5.1**). However, raloxifene and LY 117018 differ in the composition and conformation of their side chains (**Figure 5.1**) which might account for differences in their relative level of estrogen antagonism (Grese et al. 1997). In addition, while structurally different to 17β-estradiol, both raloxifene and LY117018 retain phenolic hydroxyl groups (**Figure 5.1**) that mimic the action of the C3–OH group of the phenolic A ring present in the steroid structure of 17β-estradiol (Grese et al. 1997) and could facilitate potential antioxidant activity.

Although the beneficial effects of SERMs, as part of hormone replacement therapy (HRT), in bone are well characterised on the tissue level and demonstrate clear estrogen receptor-mediated benefits to bone mass, less is known regarding the mechanism of action of these compounds in the maintenance of bone cell populations. As demonstrated in Chapter 3, the Raloxifene analogue LY117018 was shown to protect osteocytes from ovariectomy-induced apoptosis in vivo in a rat model of ovariectomy. However, further studies are required in order to determine the molecular mechanism of this protective effect.

The purpose of this study was to investigate the possibility that raloxifene or LY 117018 could mimic the anti-apoptotic effects of 17β-estradiol, presented in the previous chapter, by acting as anti-oxidants during oxidative stress-engendered induction of apoptotic cell death in MLO-Y4 osteocyte-like cells.

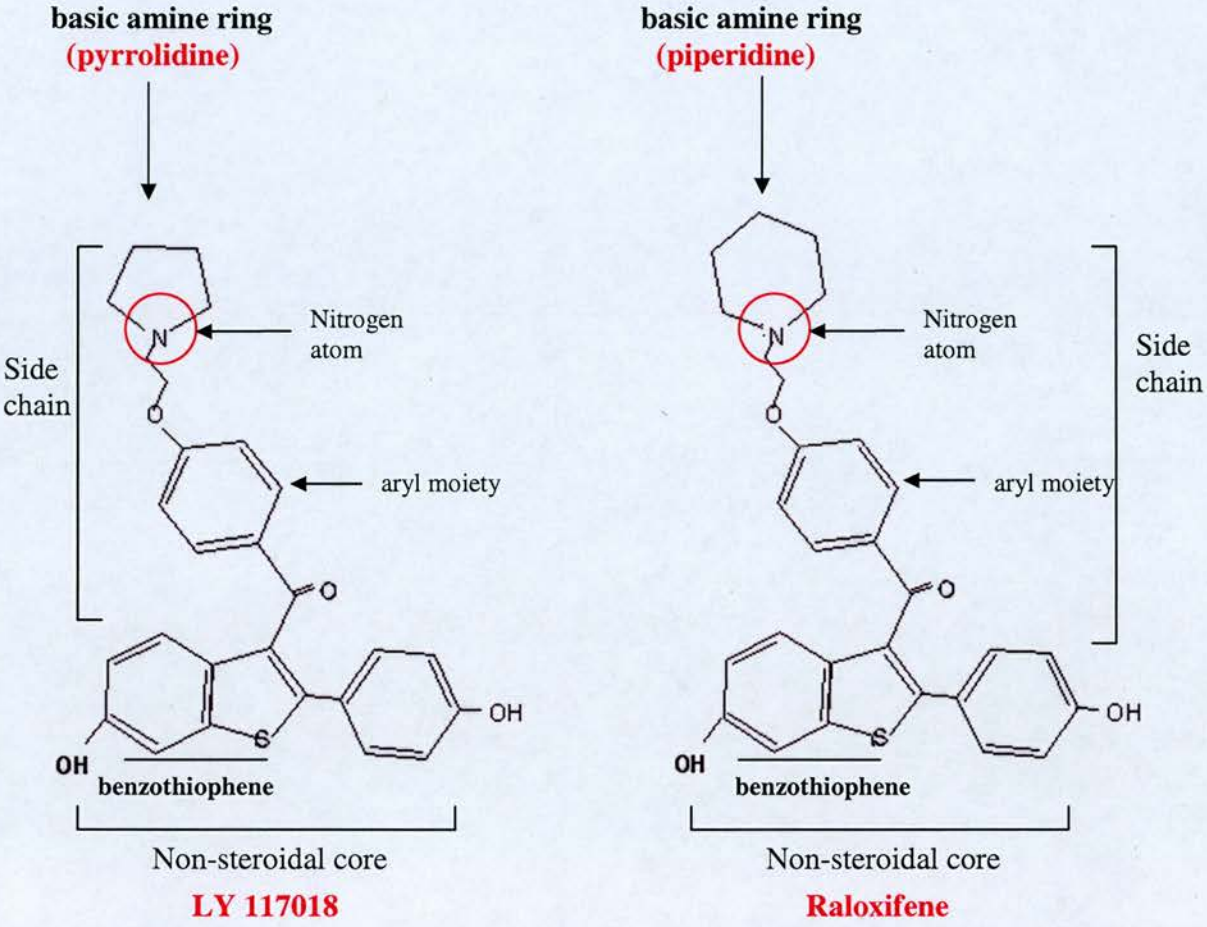


Figure 5.1. Basic chemical structure of the SERMs LY 117018 and Raloxifene, which belong to the benzothiophene group. Raloxifene and LY 117018 are characterized by the presence of a non-steroidal core and a basic side chain of an aryl moiety and an amine ring. The amine ring contains a basic nitrogen atom required for antagonising the in vivo effects of estrogen in certain tissues. The nature of this amine ring in the two SERMs is different. The amine ring of LY 117018 is known as the pyrrolidine ring and is very similar to the amine ring of raloxifene, known as piperidine, lacking however one carbon atom. The topology of the side chain determines the action of SERMs (agonist /antagonist) in different tissues. The core of these non-steroidal compounds is characterised by the presence of the benzothiophene group and the phenolic hydroxyls and is the same for both LY 117018 and raloxifene.

5.2 Materials and Methods

All chemicals were purchased from Sigma-Aldrich, UK, unless otherwise stated. all tissue culture reagents were purchased from Invitrogen, UK and tissue culture well plates were purchased from Corning, UK unless otherwise stated. Tissue culture procedures were performed in a laminar flow hood (class 2) receiving HEPA-filtered air, using sterile equipment.

5.2.1 Cell culture and maintenance

The MLO-Y4 osteocyte-like cell line, the HeLa human cervical epithelial cell line and the HEK 293 human embryonic kidney epithelial cell line were cultured as described in § 4.2.1. Briefly, osteocytes were maintained in Modified Eagles Medium Alpha (α-MEM) supplemented with 5% FBS, 5% NCS, 1% P/S and 1% L-glutamine (Kato et al. 1997). The HeLa cells were cultured in α-MEM supplemented with 5% FBS, 5% NCS, 1% P/S, and 1% L-glutamine (Kousteni et al. 2001). The HEK 293 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % FBS, 1% P/S, and 1% L-glutamine (Evdokiou et al. 1999). Subculturing was performed twice weekly upon reaching 90% of confluency, maintaining the cells in the log phase of proliferation.

5.2.2 Cell treatment

MLO-Y4 osteocytes and HeLa cells were plated in growth medium at a density of 2.4 x 10^4 cells per ml on 24 multi-well plates for 24 hours prior to experimentation, as described in § 4.2.2. HEK 293 cells were plated at a density of 5 x 10^4 cells per ml in 24 multi-well plates 24 hours prior to experimentation.

Experiments were carried out a minimum of three times, and each treatment group was represented by 3 wells in each independent experiment. Cells were observed in 3 fields per well resulting in 9 fields per treatment group, as described in § 4.2.2. SERMs were diluted in 70% ethanol while H_2O_2 was directly diluted in growth medium. Control treatments represent untreated cultures. Where indicated in the figure legends, vehicle represents treatment with 70% ethanol subjected to dilutions identical to the highest concentration under study in the absence of the compound. Tissue culture plastics, either

in the form of flasks or well plates, used to maintain the MLO-Y4 cell line were coated with 0.1M collagen type I from rat tail prior to use.

5.2.3 Induction of oxidative stress

MLO-Y4 osteocytes, HeLa cells and HEK 293 cells were incubated in growth medium supplemented with H_2O_2 (30% v/v) at a concentration of 0.3mM for 2 hours in order to induce oxidative stress, as described in § 4.2.3. Apoptotic cells were monitored microscopically using nuclear morphological criteria.

5.2.4 Prevention of cell death

5.2.4.1 Selective Estrogen Receptor Modulators (SERMs)

MLO-Y4 osteocytes, HeLa cells and HEK 293 cells were pre-treated for 1 hour with the SERMs raloxifene or LY 117018 (a kid gift from Eli Lilly) at concentrations of 10^{-6}M, 10^{-7}M and 10^{-8} M diluted in 70 % ethanol, followed by H_2O_2 treatment at 0.3mM for a further 2 hours. The pre-treatment agents remained in culture during H_2O_2 induced oxidative stress.

5.2.4.2 17β-estradiol

MLO-Y4 osteocytes, HeLa cells and HEK 293 cells were pre-treated for 1 hour with 17β-estradiol (Calbiochem, UK) diluted in 70% ethanol at a concentration of 10^{-8} M, as described in § 4.2.4.1 followed by H_2O_2 treatment at 0.3mM for further 2 hours. The pre-treatment agents remained in culture during H_2O_2 induced oxidative stress.

5.2.4.3 Pure estrogen receptor antagonist ICI 182, 780

MLO-Y4 osteocytes were pre-treated for 1 hour with the pure estrogen receptor antagonist ICI 182, 780 (Tocris Cookson Ltd, UK) at a concentration of 10^{-8}M, as described in § 4.2.4.2, in the presence or absence of raloxifene or LY 117018 followed by H_2O_2 treatment used at 0.3mM for a further 2 hours. The pre-treatment agents remained in culture during H_2O_2 induced oxidative stress.

5.2.5 Inhibition of intracellular signaling proteins

5.2.5.1 MAPK inhibitors

MLO-Y4 osteocytes were pre-incubated for 1 hour with UO126 (Promega, UK) at a concentration of 20μM, as described in § 4.2.5.2 and remained in culture prior to the further addition of SERMs and also in the presence of H_2O_2.

5.2.6 Determination of apoptotic state

5.2.6.1 DAPI staining for healthy and apoptotic cell morphology

Following experimental treatments, cells were fixed in 4 % paraformaldehyde and incubated with DAPI used at 2.5 ng/ml for 20 minutes, as described in § 4.2.6.1.

5.2.7 Detection of intracellular reactive oxygen species (ROS)

The presence of intracellular reactive oxygen species (ROS) was detected as described in § 4.2.7. Briefly, H_2DCF-DA (10 μM) (Yen et al. 2001) was added to the culture medium 30 minutes prior to incubation of MLO-Y4 osteocytes, HeLa cells and HEK 293 cells with either raloxifene, LY 117018, 17β-estradiol or vitamin E. MLO-Y4 osteocytes, HeLa and HEK 293 cells were then incubated with each of these agents, all employed at 10^{-8}M, for 1 hour prior to treatment with H_2O_2 at 0.3mM for a further 2 hours. H_2DCF-DA and all pre-treatment agents in MLO-Y4 osteocytes, HeLa cells and HEK 293 cells, remained in culture during H_2O_2 treatment after which the media was removed and the cells were washed twice with PBS before re-addition of α-MEM growth medium. Live cells were monitored for 2 hours using an inverted microscope fitted with an environmental chamber to maintain temperature and 5% humidified CO_2. For analysis 3 wells per treatment and six fields per well were counted. Negative control was represented by cultures incubated in the absence of the H_2DCF-DA in order to determine the absence of auto-fluorescence in MLO-Y4 osteocytes, HeLa and HEK 293 cell cultures.

5.2.8 Statistical analysis

Statistical analysis were performed using quantitative data analysis with SPSS release 10.1 for Windows, as described in § 4.2.9 using Analysis of Variance (ANOVA), followed by the Tukey-Kramer and Dunnett post-hoc tests for comparison between the treatment groups and comparison between each treatment group with the experimental control, respectively (only when the significance of the ANOVA was $p<0.05$). Results were expressed as means ± S.E. Results are considered to be statistically significant when $p<0.05$ denoted by *; $p<0.01$ denoted by ** and $p<0.001$ denoted by *** when experimental treatment was compared to H_2O_2 treatment, and $p<0.05$ denoted by †, $p<0.01$ denoted by †† and $p<0.001$ denoted by ††† when experimental treatment was compared to the non-treated control.

5.3 Results

5.3.1 Raloxifene and LY 117018 prevent H_2O_2-induced apoptosis in MLO-Y4 osteocytes.

Dose response studies were used to identify concentrations of raloxifene and LY 117018 that did not increase apoptosis above the control levels. Mean percentages of apoptotic osteocytes following treatment of osteocytes with raloxifene and LY 117018 used at concentrations of 10^{-6}M , 10^{-7}M and 10^{-8}M were not statistically different to the control cultures ($p>0.05$) (**Figure 5.2A and 5.2B**). Estimation of apoptotic osteocytes based on morphological criteria showed that raloxifene or LY117018, at all concentrations used, significantly reduced the pro-apoptotic effect of H_2O_2 at 0.3mM following two hours of incubation in culture (**Figure 5.2A, 5.2B, 5.3**). The lowest concentration of 10^{-8}M for both raloxifene and LY 117018, which was comparable to the optimal anti-apoptotic concentration of 17β-estradiol (**Figure 5.2C**), was chosen to pre-treat osteocytes prior to addition of 0.3mM H_2O_2 in subsequent experiments.

5.3.2 The estrogen receptor antagonist ICI 182 780 does not prevent the anti-apoptotic effects of raloxifene or LY 117018.

As described in chapter 1, estrogen receptor antagonist ICI 182, 780 was employed in order to determine whether the observed anti-apoptotic effects of the above mentioned SERMs on MLO-Y4 osteocytes was mediated via the nuclear estrogen receptor. In a similar way to 17β-estradiol (as described in § 4.3.5), the anti-apoptotic effect of both raloxifene and LY117078 was not blocked by pre-incubation of osteocyte cultures with ICI 182,780 at 10^{-8}M for 1 hour prior to the addition of the SERMs (**Figure 5.4**). Furthermore, pre-treatment of osteocytes with ICI 182 780 in the absence of the SERMs significantly reduced H_2O_2- induced apoptosis indicating that the ICI 182 780 compound has anti-apoptotic activity in this model system as described in § 4.3.5 (**Figure 5.4**).

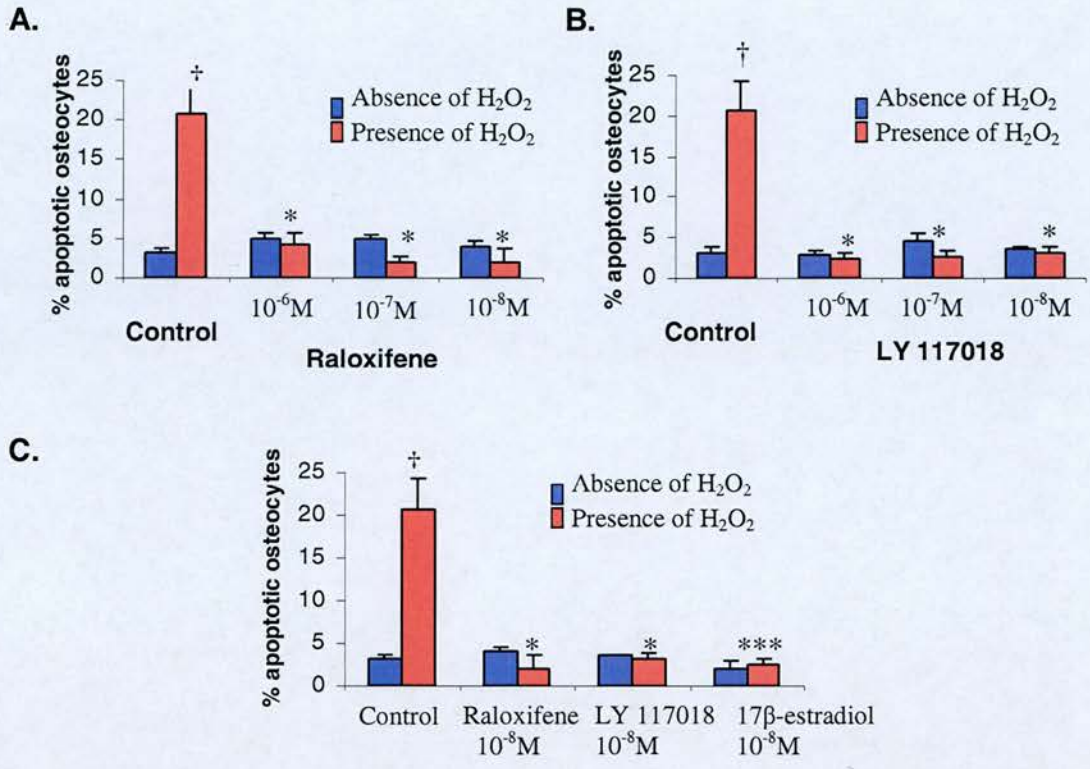


Figure 5.2. Raloxifene and LY 117018 prevent osteocyte apoptosis induced by H_2O_2 in a concentration-dependent manner. Osteocytes were incubated with **A.** raloxifene and **B.** LY 117018 at 10^{-6}M-10^{-8}M for 1 hour prior to H_2O_2 treatment for a further 2 hours. Mean percentages of apoptotic osteocytes were not significantly different compared to control cultures following treatment with **A.** raloxifene and **B.** LY 117018 at all concentrations used. All concentrations of **A.** raloxifene and **B.** LY 117018 reduced the percentages of apoptotic osteocytes induced by H_2O_2 treatment to levels similar to control. **C.** Pre-incubation of osteocytes with raloxifene and LY 117018 at 10^{-8}M for 1 hour in the presence of H_2O_2 prevented H_2O_2-induced osteocyte apoptosis to a similar extent as 17β-estradiol. Graphs represent mean percentages of apoptotic osteocytes ± SE., estimated by DAPI nuclear staining and fluorescent microscopy. (*** = $p< 0.0001$, * = $p< 0.05$ compared to H_2O_2 treatment, † = $p<0.05$ compared to control). Control cultures represent untreated cultures. Vehicle cultures for raloxifene and LY 117018 at 10^{-6}M were similar to control in percentages of apoptotic HeLa cells (4.61 ± 1.57 % S.E., $p>0.05$; 4.4 ± 0.93 % S.E., $p>0.05$).

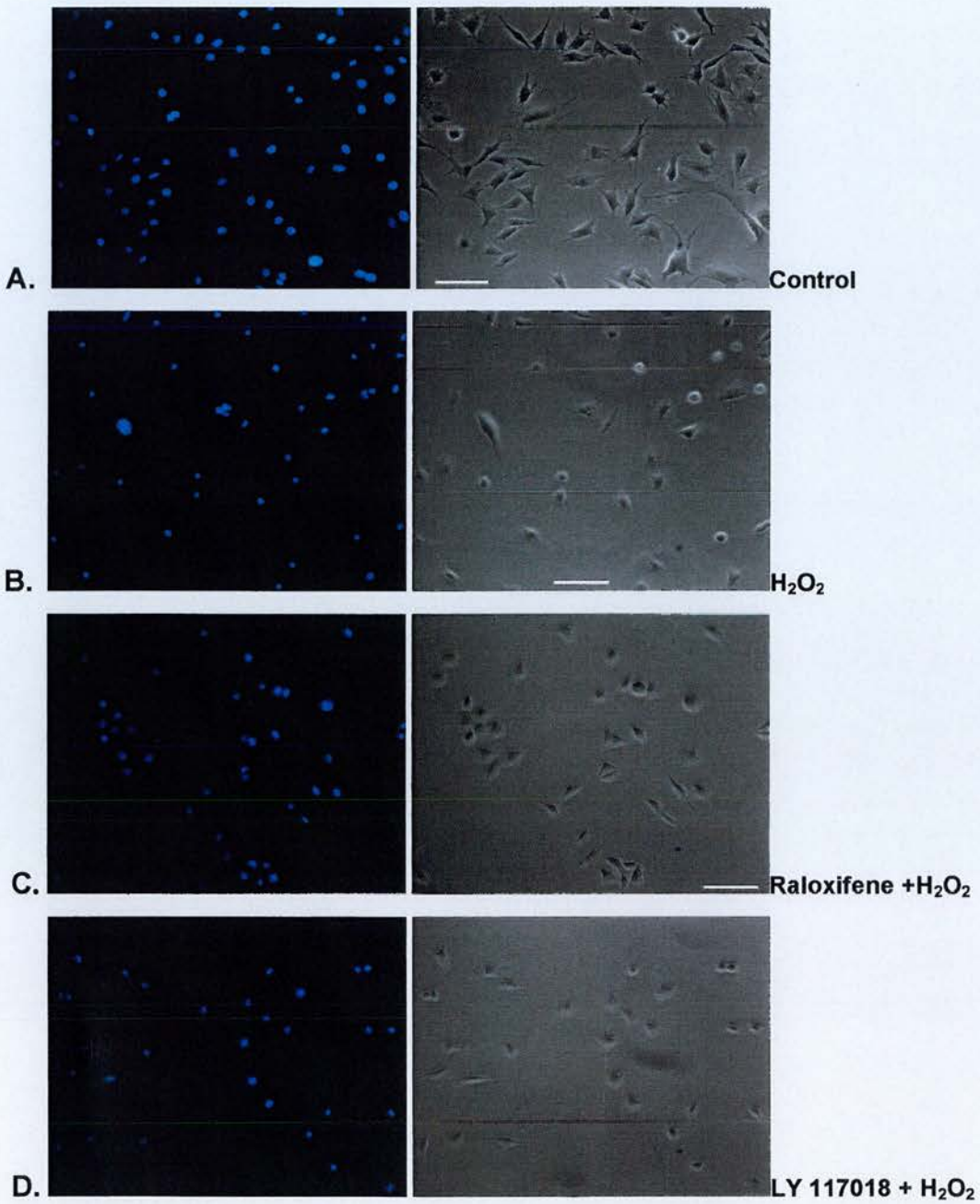


Figure 5.3. Raloxifene and LY 117018 prevent H_2O_2-induced apoptosis in MLO-Y4 osteocyte cultures. Representative images of osteocytes in culture treated with either raloxifene or LY 117018 for 1 hour followed by H_2O_2 treatment for a further two hours and stained with DAPI. H_2O_2 induced osteocyte apoptosis (**B**), which was reversed following administration of either raloxifene (**C**) or LY 117018 (**D**). Bar = 10 μm.

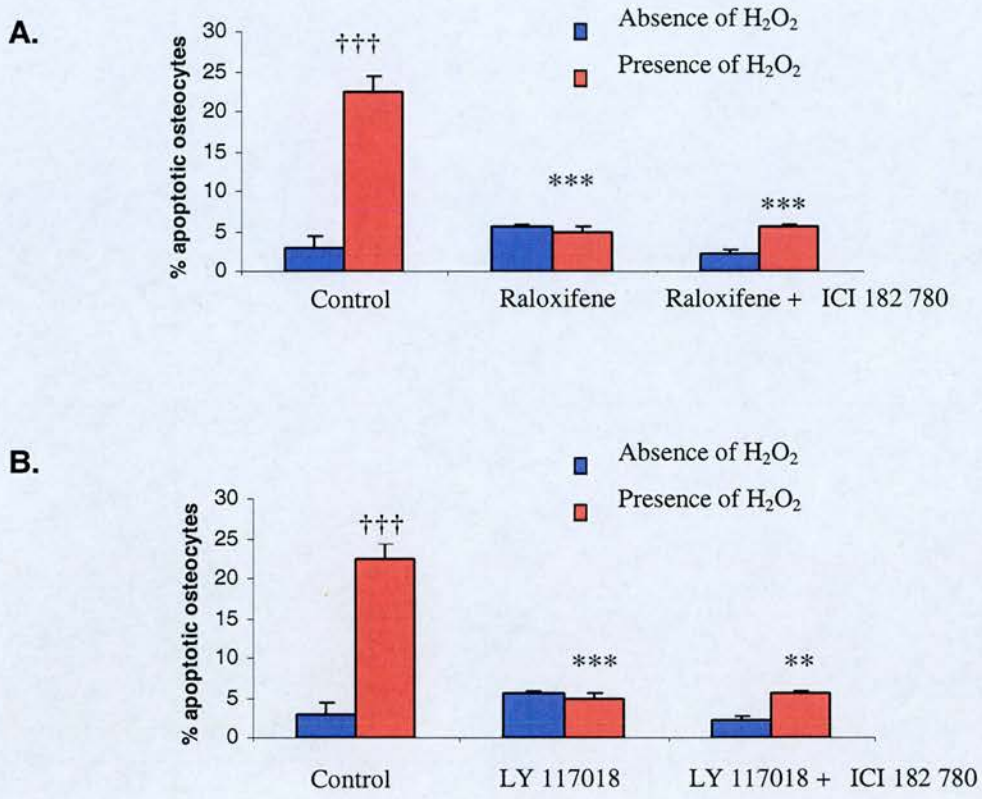


Figure 5.4. ICI 182 780 does not prevent the protective effects of raloxifene and LY 117018 on MLO-Y4 osteocyte apoptosis induced by H_2O_2. In order to determine whether the anti-apoptotic effects exerted by SERMs were mediated via the nuclear estrogen receptor osteocytes were pre-treated with the pure estrogen receptor antagonist ICI 182 780 at 10^{-8} M for 1 hour prior to the addition of **A.** raloxifene and **B.** LY 117018 for 1 hour in the presence or absence of H_2O_2 at 0.3mM for a further 2 hours. Pre-treatment of cells with ICI 182,780 did not inhibit the anti-apoptotic effects of **A.** raloxifene and **B.** LY 117018. Cells were stained with DAPI and examined by fluorescence microscopy. Graphs represent mean percentages of apoptotic osteocytes ± SE. (*** =$p<0.0001$, ** = $p< 0.001$, compared to H_2O_2 treatment, ††† =$p<0.001$ compared to control).

5.3.3 Raloxifene and LY 117018 prevent H_2O_2-induced apoptosis in ER-negative HeLa cells

Administration of H_2O_2 at the dose of 0.3mM induced apoptosis in HeLa cells over a 2 hour incubation period, as described in § 4.3.6. Administration of raloxifene or LY 117018 for 1 hour at concentrations of 10^{-6}M, 10^{-7}M and 10^{-8}M did not increase the levels of apoptosis above control levels ($p>0.05$), (**Figure 5.5A and 5.5B**). Pre-treatment of HeLa cells with raloxifene and LY117018 for 1 hour at all concentrations used significantly reduced the percentage of H_2O_2 induced apoptosis following two hours of incubation in culture ($p<0.001$, $p= 0.001$, $p=0.02$, respectively for raloxifene), ($p=0.02$, $p= 0.008$, $p=0.0015$, respectively for LY 117018), (**Figure 5.5A and 5.5B**). The dose of 10^{-8} M for both raloxifene and LY 117018, comparable to the optimal anti-apoptotic concentration of 17β-estradiol (**Figure 5.5C**), was employed in subsequent treatments.

5.3.4 Raloxifene and LY 117018 prevent H_2O_2-induced apoptosis of the ER-negative HEK 293 cells

To further investigate the potential contribution of the estrogen receptor in the anti-apoptotic effects of raloxifene or LY 117018 against H_2O_2 induced death, the HEK 293 cell line that lacks the ER was employed (Kahlert et al. 2000, Thomas et al. 2005). The dose of 0.3mM H_2O_2, which was used to characterise oxidative stress in MLO-Y4 osteocytes and HeLa cells, was also employed in the HEK 293 cell line as mentioned in § 4.3.7. Pre-treatment of HEK 293 cells with raloxifene (**Figure 5.6A**) or LY 117018 (**Figure 5.6B**) for 1 hour at doses of 10^{-6}M, 10^{-7}M and 10^{-8}M all significantly reduced the percentage of H_2O_2 induced apoptosis ($p<0.05$ for 10^{-6}M and 10^{-7}M and $p<0.001$ for 10^{-8}M for raloxifene; $p< 0.05$ for 10^{-6}M, 10^{-7}M and 10^{-8}M for LY 117018).

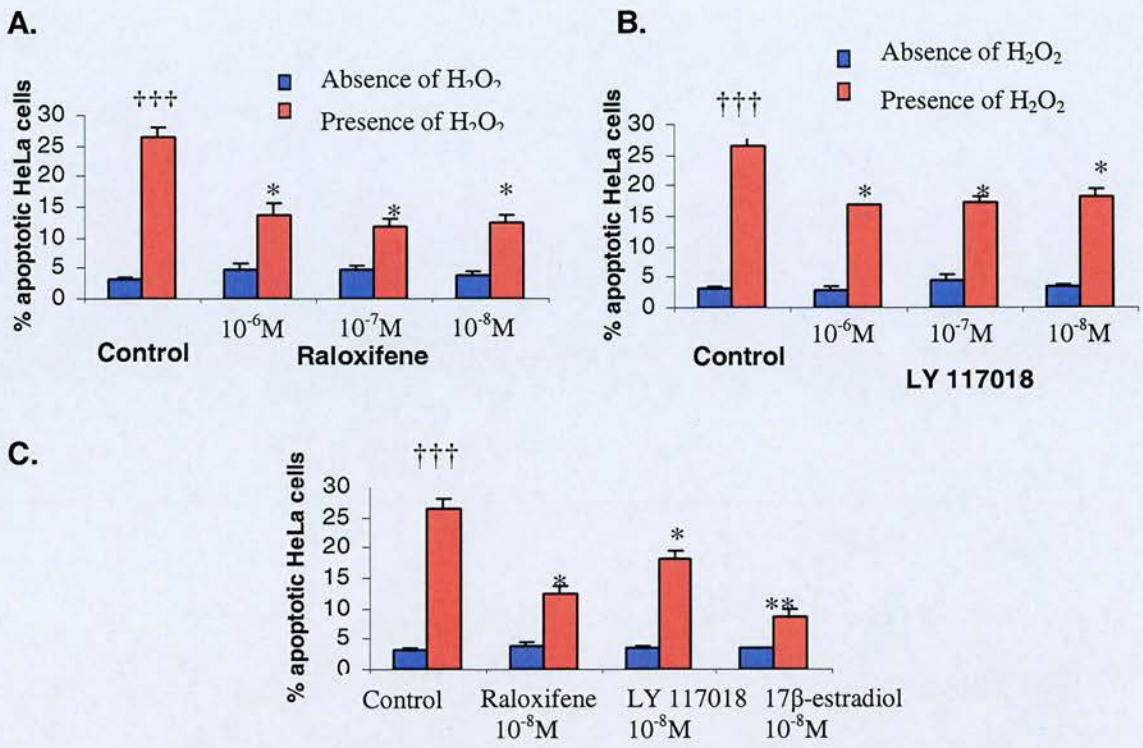
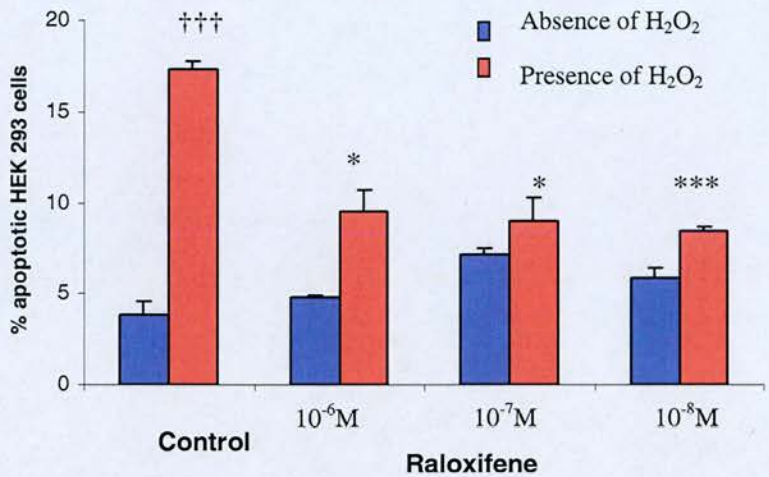


Figure 5.5. Raloxifene and LY 117018 prevent HeLa cell apoptosis induced by H_2O_2. HeLa cells were incubated with **A.** raloxifene and **B.** LY 117018 at 10^{-6} M, 10^{-7} M and 10^{-8} M for 1 hour prior to H_2O_2 treatment for a further 2 hours. **C.** Pre-incubation of HeLa cells with raloxifene and LY 117018 at 10^{-8}M for 1 hour in the presence of H_2O_2 prevented H_2O_2-induced apoptosis in HeLa cells to a similar extent as 17β-estradiol Cells were stained with DAPI and examined with fluorescence microscopy Graphs represent mean percentages of apoptotic HeLa cells ± SE. (** = $p< 0.001$, * = $p< 0.05$ compared to H_2O_2 treatment, ††† = $p<0.0001$, compared to control). Control cultures represent untreated cultures. Vehicle cultures for raloxifene and LY 117018 at 10^{-6}M were similar to control in percentages of apoptotic HeLa cells (4.07 ± 0.91 % S.E., $p>0.05$; 2.88 ± 0.35 % S.E., $p>0.05$).

A.



B.

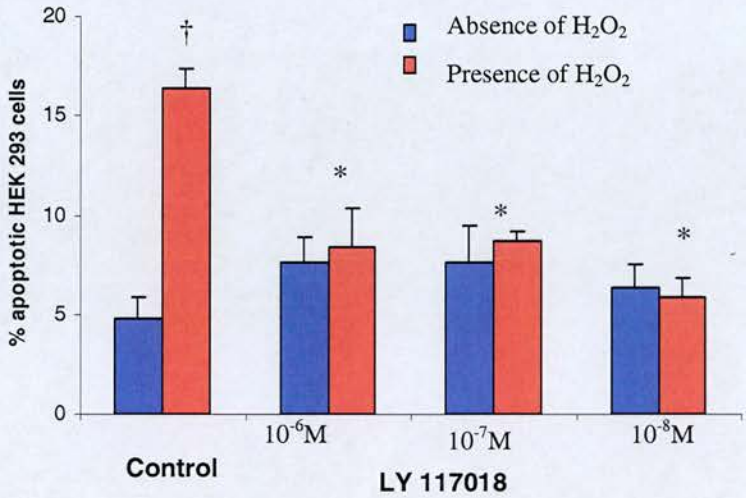


Figure 5.6. Raloxifene and LY 117018 prevent HEK 293 cell apoptosis induced by H_2O_2. HEK 293 cells were incubated with **A.** raloxifene and **B.** LY 117018 at 10^{-6} M, 10^{-7} M and 10^{-8} M for 1 hour prior to H_2O_2 treatment for a further 2 hours. Cells were stained with DAPI and examined with fluorescence microscopy Graphs represent mean percentages of apoptotic HEK 293 cells ± SE. (*** = $p< 0.0001$, * = $p< 0.05$ compared to H_2O_2 treatment, ††† = $p<0.0001$, †= $p<0.01$, compared to control). Control cultures represent untreated cultures. Vehicle cultures for raloxifene at 10^{-6}M were similar to control in percentages of apoptotic HEK 293 cells.

5.3.5 ERK 1/2 inhibition does not block the protective effects of SERMs on MLO-Y4 osteocytes.

As described in chapter 4 (§ 4.2.5.2), the MEK 1/2 inhibitor UO126 was used in order to determine whether the saving effect of raloxifene or LY 117018 was mediated via receptor activation of MAP Kinase signalling pathways. Pre-treatment of osteocytes with UO126 at 20μM prior to the addition of raloxifene or LY117018 for 1 hour did not inhibit the anti-apoptotic effects of the raloxifene and LY 117018 against the H_2O_2 treatment (**Figure 5.7**).

5.3.6 Raloxifene or LY 117018, compounds that contain the –OH group, reduced the H_2O_2-induced ROS production in MLO-Y4 osteocytes

As described in § 4.3.12, treatment of osteocytes with H_2O_2 for 2 hours resulted in a significant increase in the proportion of ROS positive cells compared to the control ($p=0.002$). Pre-treatment of osteocytes with raloxifene or LY 117018 for 1 hour, at a concentration of 10^{-8}M, significantly reduced the percentage of H_2O_2-induced ROS positive cells to levels similar to those seen in control cultures, as did the potent anti-oxidant Vitamin E at 10^{-8}M (**Figure 5.8**). In contrast, pre-incubation of osteocytes with mestranol at 10^{-8} M concentration failed to reduce the number of ROS positive cells, as described in § 4.3.12, (**Figure 5.8**).

5.3.7 Raloxifene or LY 117018 reduced the H_2O_2-induced ROS production in HeLa cells

As described in chapter § 4.3.13, treatment of HeLa cells with H_2O_2 for 2 hours resulted in a significant increase in the proportion of ROS positive cells compared to the control ($p=0.01$). Pre-treatment of HeLa cells with raloxifene or LY 117018 for 1 hour used at a concentration of 10^{-8}M significantly reduced the percentage of H_2O_2-induced ROS positive osteocytes to levels similar to those seen in control cultures ($p=0.01$), (**Figure 5.10**).

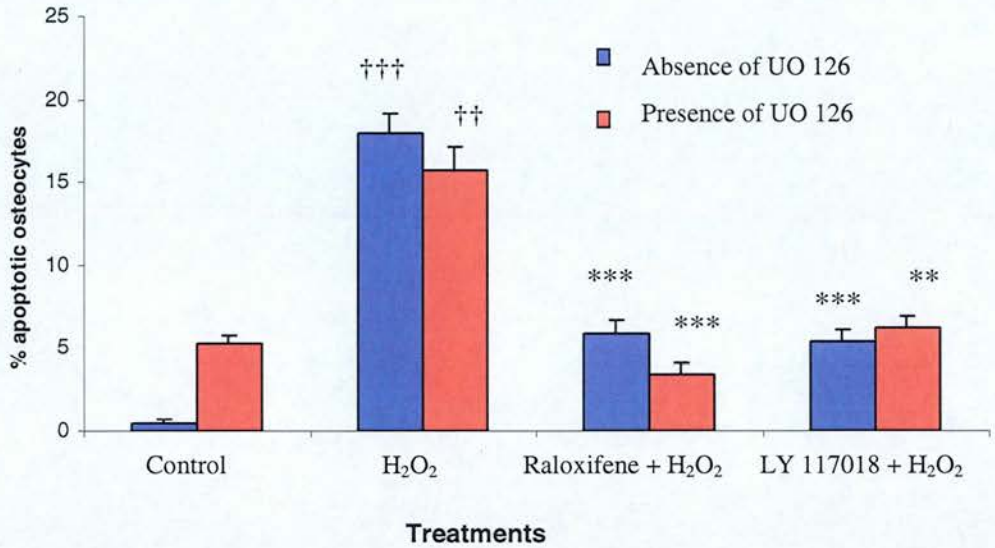


Figure 5.7. The MEK ½ inhibitor U0126 does not inhibit the anti-apoptotic effects of raloxifene or LY 117018. MLO-Y4 osteocytes were incubated with UO126 for 30 minutes at 20 μM prior to the addition of raloxifene and LY 117018 for 1 hour in the presence or absence of H_2O_2 at 0.3mM for a further 2 hours. UO126 did not prevent MLO-Y4 osteocyte apoptosis induced by H_2O_2 treatment and did not block the protective effect of raloxifene and LY 117018 on MLO-Y4 osteocytes. Cells were stained with DAPI and examined by fluorescence microscopy. Graphs represent mean percentages of apoptotic osteocytes ± SE. (*** =$p<0.0001$, ** = $p< 0.001$, compared to H_2O_2, †††=$p<0.0001$, ††=$p<0.001$, compared to control). Control cultures represent untreated cultures and are similar to vehicle (DMSO) cultures in percentages of apoptotic osteocytes.

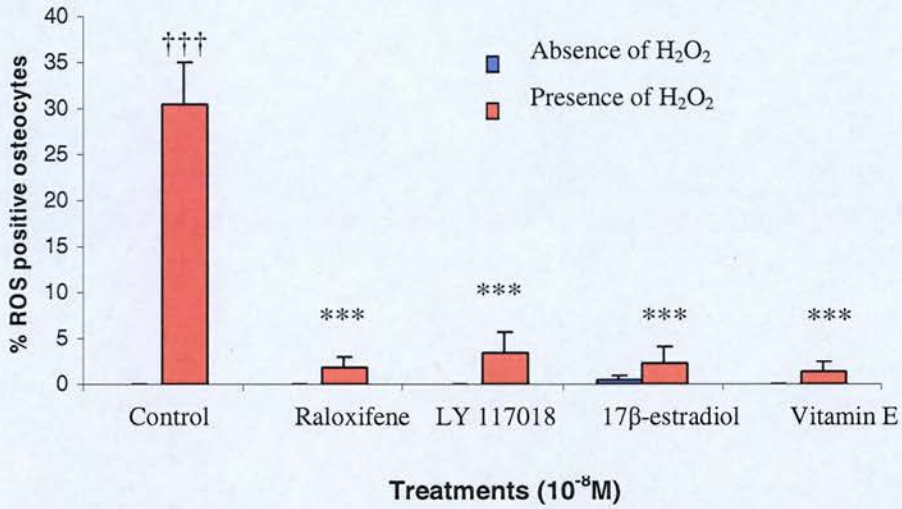


Figure 5.8. Raloxifene or LY 117018, which display the C3-OH moiety in their structure, prevent H_2O_2–induced generation of ROS in MLO-Y4 osteocytes. MLO-Y4 osteocytes were incubated with H_2DCF-DA for 30 min at 10μM and then pre-treated with raloxifene, LY 117018, 17β-estradiol and vitamin E at 10^{-8}M for 1 hour in the presence or absence of H_2O_2 at 0.3mM, for a further 2 hours. The percentage of ROS positive osteocytes was significantly increased compared to control following treatment with H_2O_2. Pre-treatment of MLO-Y4 osteocytes with raloxifene, LY 117018, 17β-estradiol and vitamin E significantly reduced the percentage of ROS positive osteocytes following treatment with H_2O_2. Cells were examined by fluorescence microscopy. Graphs represent mean percentages of ROS positive osteocytes ± SE. (*** = $p< 0.0001$, compared to H_2O_2, ††† =$p<0.0001$ compared to the control). Control cultures represent untreated cultures.

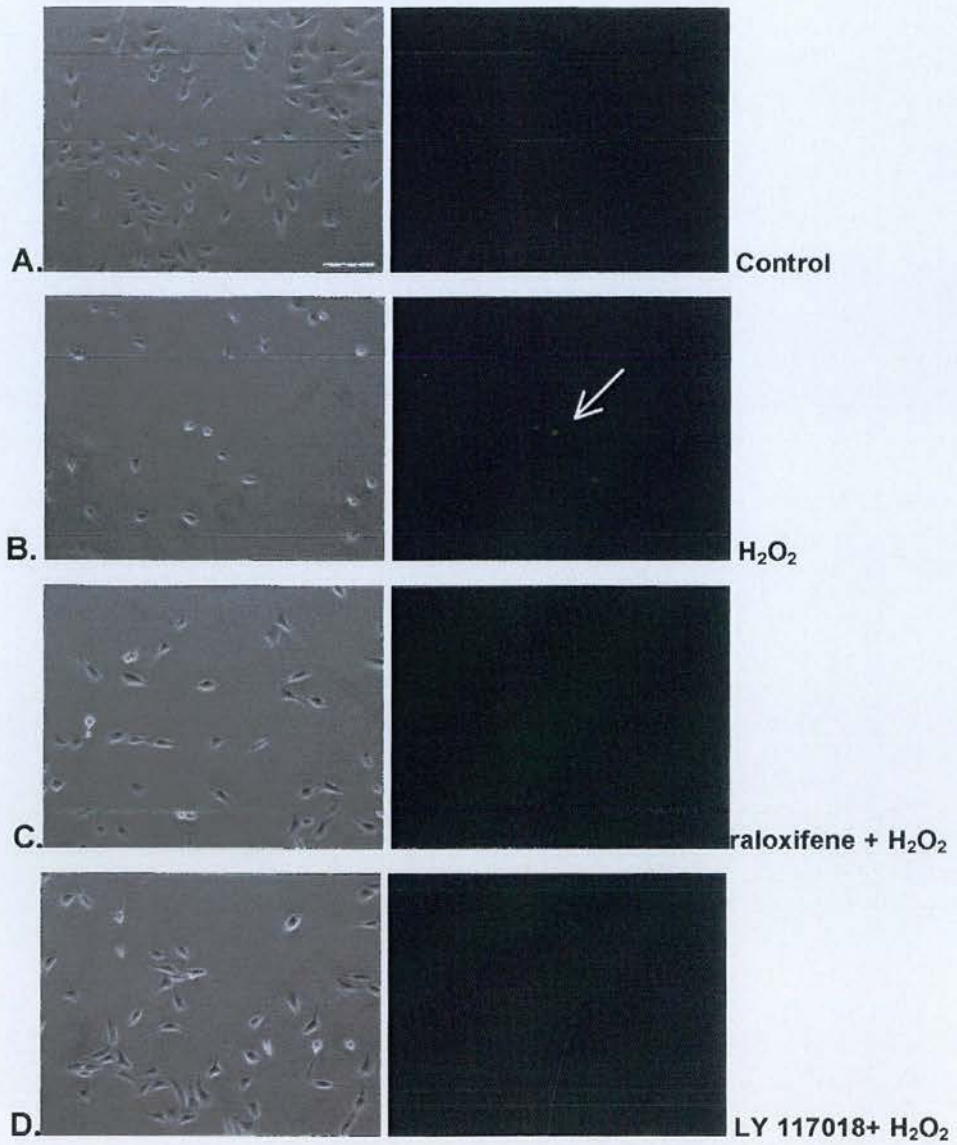


Figure 5.9. Raloxifene and LY 117018 reduced the H_2O_2-induced ROS production in MLO-Y4 osteocytes. MLO-Y4 osteocytes were incubated with H_2DCF-DA for 30 min and then pre-treated with raloxifene and LY 117018 for 1 hour prior to H_2O_2 treatment for a further two hours. **A.** control cultures, **B.** H_2O_2-treated cultures, **C.** cultures treated with raloxifene in the presence of H_2O_2, **D.** cultures treated with LY 117018 in the presence of H_2O_2. White arrow indicates a ROS-positive (FITC) osteocyte. Bar = 10μm.

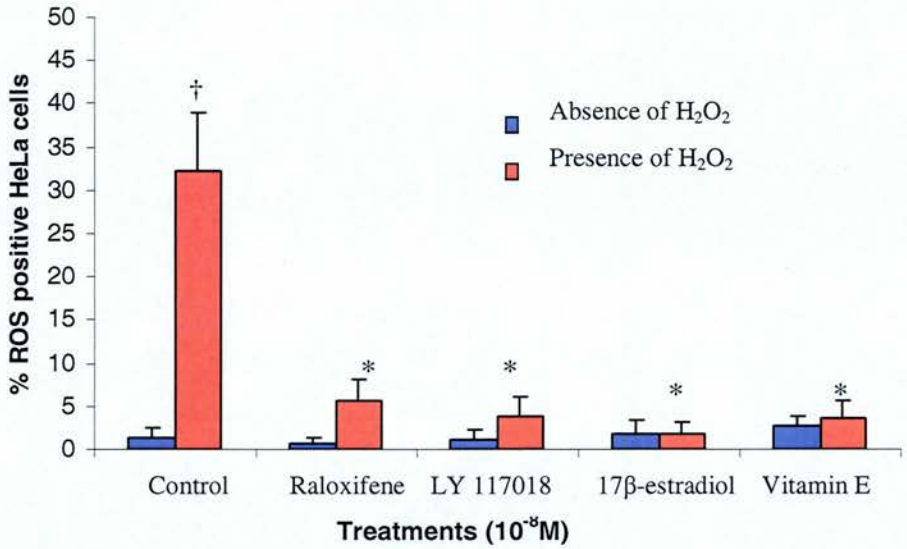


Figure 5.10. Raloxifene or LY 117018 prevent H_2O_2–induced production of ROS in HeLa cells. HeLa cells were incubated with H_2DCF-DA for 30 min at 10μM prior to the addition of raloxifene, LY 117018 and 17β-estradiol for 1 hour in the presence or absence of H_2O_2 at 0.3mM, for a further 2 hours. The percentage of ROS positive HeLa cells was significantly increased compared to control following treatment with H_2O_2. Pre-treatment of HeLa cells with raloxifene, LY 117018 and 17β-estradiol used at a concentration of 10^{-8}M, significantly reduced the percentage of ROS positive HeLa cells following treatment with H_2O_2. Cells were examined by fluorescence microscopy. Graphs represent mean percentages of ROS positive HeLa cells ± SE. (* = $p< 0.05$, compared to H_2O_2, † = $p<0.05$ compared to control). Control cultures represent untreated cultures.

5.3.8 Raloxifene or LY 117018 that contain the –OH group reduced the H_2O_2-induced production of ROS in HEK 293 cells

The proportion of ROS positive cells was significantly increased within 2 hours of treatment of HEK 293 cells with H_2O_2 ($p=0.04$), (**Figure 5.11**). Pre-treatment of HEK 293 cells with raloxifene or LY 117018, at a concentration of 10^{-8}M, significantly reduced the proportion of ROS positive HEK 293 cells induced by H_2O_2 treatment ($p=0.019$ and $p= 0.018$, respectively) (**Figure 5.11**).

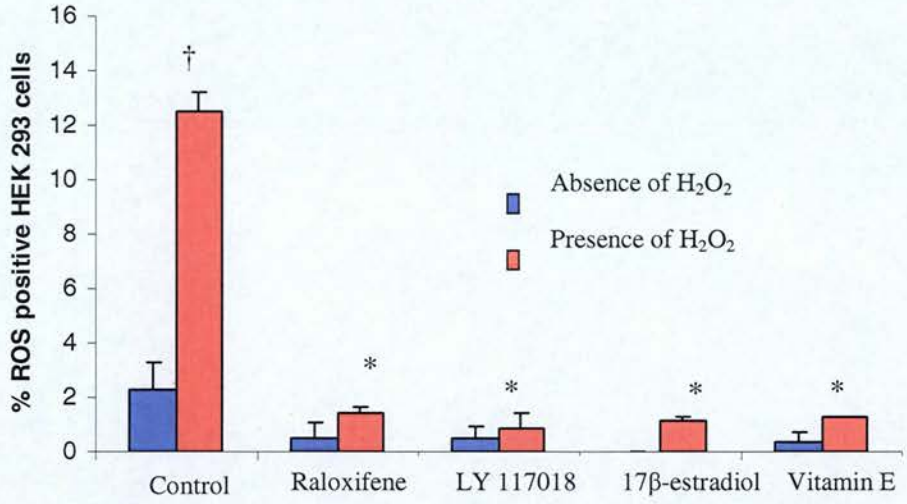


Figure 5.11. Raloxifene or LY 117018 that display the C3-OH moiety in their structure prevent H_2O_2–induced generation of ROS in HEK 293 cells. HEK 293 cells were incubated with H_2DCFH-DA for 30 min and then pre-treated with raloxifene, LY 117018, 17β-estradiol or vitamin E for 1 hour prior to addition of H_2O_2 for a further 2 hours. Pre-treatment with raloxifene, LY 117018, 17β-estradiol or vitamin E reduced the percentage of ROS positive cells induced by H_2O_2 treatment to levels similar control. Cells were examined with fluorescence microscopy. Graphs represent mean percentages of ROS positive HEK 293 cells ± SE. (* = $p< 0.05$, compared to H_2O_2, † =$p<0.05$ compared to control). Control cultures represent untreated cultures.

5.4 Discussion

Data presented in chapter 4 have demonstrated that 17β-estradiol was capable of preventing osteocyte apoptosis in response to oxidant attack in vitro. Data provided in this study demonstrate that both raloxifene and LY 117018 are equally effective as 17β-estradiol in reducing the pro-apoptotic signals induced by H_2O_2 administration on the MLO-Y4 osteocytic population in vitro.

Pre-treatment of osteocyte cultures with raloxifene and LY 117018 reduced the proportion of osteocytes displaying membrane blebbing and cytoplasmic and chromatin condensation induced by H_2O_2 treatment (**Figures 5.2 and 5.3**). Previous studies have demonstrated that, in response to alternative forms of death such as etoposide, raloxifene was not capable of protecting MLO-Y4 osteocytic cells from apoptosis (Kousteni et al. 2003) indicating that the protective effects exerted by raloxifene could be related to specific death-inducing stimuli.

Investigation of the ERK 1/2 pathway revealed that pre-incubation of MLO-Y4 osteocytes with the ERK1/2 inhibitor UO 126 did not abrogate the anti-apoptotic effects of neither raloxifene nor LY 117018 on osteocytes (**Figure 5.7**). Furthermore, Kousteni et al have demonstrated the inability of raloxifene to activate the ERK1/2 pathway in MLO-Y4 osteocytes (Kousteni et al. 2003) suggesting that the ERK1/2 pathway might not be implicated in the prevention of oxidative stress by the SERMs raloxifene and LY 117018 in osteocytes, as also indicated in this chapter. These findings are also in agreement with results from chapter 4, which demonstrated that 17β-estradiol did not utilise the ERK1/2 pathway in order to prevent H_2O_2-induced osteocyte apoptosis under identical culture conditions.

At the molecular level raloxifene has been shown to exert its effects both through classical estrogen receptor mechanisms (Taranta et al. 2002) as well as non classical estrogen receptor mechanisms (Yang et al. 1996, Balfour et al.1998). In order to elucidate the importance of the estrogen receptor in the SERM mediated response, the

pure estrogen receptor antagonist ICI 182 780 and the ER-negative HeLa (Rago et al. 2004, Carpino et al. 2004) and HEK 293 cell lines (Kahlert et al. 2000, Thomas et al. 2005) were employed. Both raloxifene and LY 117018 were capable of inhibiting oxidant-induced apoptosis in the MLO-Y4 osteocytes even in the presence of the ICI 182 780 (**Figures 5.4A and 5.4B**). In addition, exertion of anti-apoptotic effects by raloxifene or LY 117018 in the ER negative HeLa (**Figures 5.5**) and HEK 293 cells (**Figures 5.6**) in response to H_2O_2 attack further supported the activation of an ER-independent mechanism of apoptosis blockade, as previously suggested for 17β-estradiol in chapter 4.

Studies outlined in the previous chapter have demonstrated that the ability of the estrogenic compounds to exert protective effects against oxidant attack in MLO-Y4 osteocytes might be associated with the presence of the C3-OH group in their phenolic A ring. Examination of the structures of raloxifene and LY 117018 revealed that both compounds also contained a hydroxyl group associated with their phenolic moiety (**Figure 5.1**) in principle capable of mimicking the steroid A-ring phenolic hydroxyl group of 17β-estradiol. In a similar manner to 17β-estradiol, both SERMs were capable of reducing the generation of ROS induced in response to H_2O_2 in osteocytic cultures, as detected by the use of the H_2DCF-DA dye (described in Chapter 4), (**Figures 5.8 and 5.9**). This finding indicates a possible antioxidant mechanism of action exerted by raloxifene or LY 117018 in osteocytes which might be associated with the presence of the hydroxyl moiety in their structures.

Several lines of evidence exist in the literature to support an antioxidant function of SERMs, as reviewed in Chapter 2. For example, raloxifene has been shown to prevent apoptosis induced by H_2O_2 in neurons (Biewenga et al. 2005) while it was also involved in the reduction of atherosclerotic lesions by reducing the release of ROS from vascular cells and increasing the bioavailability of nitric oxide (NO) (Wassmann et al. 2002). In addition, raloxifene treatment on plasma collected from postmenopausal women that had been pre-exposed to $CuSO_4$ in vitro was shown to prevent oxidation of low density

lipoproteins (LDLs) more effectively than estrogen or tamoxifen used in the same study (Arteaga et al. 2003). However, in this study reduction in the levels of ROS in the presence of either LY 117018 or raloxifene was comparable to that induced by 17β-estradiol pointing to similar free radical scavenging properties between SERMs and 17β-estradiol in osteocytes.

Data provided in this thesis have shown that LY 117018 exerted protective effects on osteocytes against OVX-induced apoptosis in vivo while in this chapter both raloxifene and LY 117018 were shown to prevent osteocyte apoptosis induced by H_2O_2 in vitro. Furthermore, this study has identified a novel action of SERMs as free radical scavengers which might account for their anti-apoptotic effects in osteocytes.

The presence of the C3–OH group identified in this chapter to be responsible for the novel function of SERMs as antioxidants in osteocytes, could contribute further to our understanding of the molecular design criteria required for the production of new SERMs that would maintain bone quality and prevent bone diseases.

CHAPTER 6

The influence of 17β-estradiol and mechanical stimulation on osteocyte apoptosis and bone viability in human trabecular bone.

Abstract

Data presented in previous chapters have demonstrated the beneficial effects of 17β-estradiol administration on the viability and apoptotic death of murine osteocytes *in vitro* and rat osteocytes *in vivo*. This pilot study attempted to investigate the effect of 17β-estradiol on the viability of osteocytes in human cancellous bone explants *ex* vivo in the presence or absence of mechanical loading using a 3D bioreactor system (Zetos^{TM}).

Bone cores obtained from the human femoral head were incubated in the bioreactor system for 27 days and subjected either to disuse conditions (Unloaded), physiological loading regime on a daily basis (Loaded), disuse conditions in the presence of 17β-estradiol (Unloaded+E_2) and mechanical loading in the presence of 17β-estradiol (Loaded+E_2). Outcome measures following 27 days in culture included osteocyte viability based on LDH activity, osteocyte apoptosis based on situ nick translation technique and histomorphometric indices based on the use of the BIOQUANT OSTEO histomorphometry software.

Results suggested that application of either mechanical stimulation or 17β-estradiol alone increased the Bone Formation Rate (BFR/BS), the mineral apposition rate (MAR) and the proportion of pseudo double-labelled surfaces compared to the unloading conditions. Furthermore, application of mechanical loading at physiological levels improved osteocyte viability and significantly reduced osteocyte apoptosis relative to that seen under disuse conditions. In contrast, administration of mechanical stimulation and 17β-estradiol or 17β-estradiol alone to the unloaded samples did not reduce osteocyte apoptosis compared to the disuse conditions.

These data have indicated that application of physiological loading or administration of 17β-estradiol using the 3D bioreactor system increased evidence of bone formation in human trabecular bone.

6.1 Introduction

It is well documented in the literature that osteocytes sense the mechanical strains produced in their local environment and produce a number of molecules such as nitric oxide (NO) (Pitsillides et al. 1995, Klein Nulend et al. 1995), prostaglandin (PGE_2) (Ajubi et al. 1999) and collagen type I gene expression (Sun et al. 1995). Production of these molecules by osteocytes leads to subsequent changes in the function of the bone effector cells, regulating therefore the bone remodelling process in order to allow the tissue to adapt efficiently to the mechanical requirements (Burger et al. 1995, Lanyon 1993, Mullender and Huiskes 1997).

Studies have reported an age-related decline in osteocyte viability (Frost 1960, Dunstan et al. 1993, Qiu et al. 2005) which may result in impaired detection of mechanical strain and microdamage, leading to inappropriate bone remodelling, accumulation of microdamage (Mori et al. 1997), loss of micro-architecture and increased fracture risk (Burr et al. 1985, Parfitt 1993). However, the mechanism by which osteocytes might be regulating the remodelling process is not understood yet. A line of evidence is available to suggest that the death of osteocytes through apoptosis might be providing such signals (Noble et al. 1997, Verborgt et al. 2000, Noble et al. 2003, Aguirre et al. 2006) since osteocyte death has been associated with increased resorptive activity in response to estrogen deficiency (Tomkinson et al. 1997, 1998) and chronic use of glucocorticoids (Weinstein et al. 1998). In addition, it has been hypothesised that a U-shaped relationship exists between the survival of osteocytes and the strains produced in their local environment such that low or excessive strains lead to osteocyte apoptosis following microdamage (Noble et al. 2003). This hypothesis might provide evidence for a possible causal mechanism by which signals derived from apoptotic osteocytes can regulate bone architecture (Noble et al. 2003).

Studies have shown that the application of mechanical loading that produced strains within the physiological range appeared to preserve osteocyte viability compared to the unloaded conditions in vivo (Noble et al. 2003) and ex vivo (Lozupone et al. 1996) as

well as to provide an osteogenic stimulus required by the bone tissue in order to maintain its structure and function (Frost 1988, Lanyon 1996, Robling 2000).

Evidence has suggested that the adaptive response of bone to mechanical stimulation is negatively affected by conditions such as estrogen loss and ageing. In particular, studies have shown that the presence of estrogen enhanced the osteogenic response of rat ulna to mechanical stimulation (Cheng et al. 1994, 1996, 1997) while reduced expression and activity of the ERα has been shown to limit the anabolic effects of mechanical stimulation on murine bone (Lee et al. 2003). Further studies are required in order to characterise the physiological significance of the relationship between 17β-estradiol and mechanical stimulation in the human bone that may lead to the development of therapeutic regimes against post-menopausal osteoporosis. However, this task has proved difficult to undertake due to the inability to obtain human bone explants before and after exercise and the difficulty in maintaining viable cultures of bone explants ex vivo (Jones et al. 2003).

In this study, a novel 3D bioreactor system (Zetos ^{TM}) that enables the long term ex vivo culture of human cancellous bone explants in a well-controlled mechanical environment (Jones et al. 2003, Davies et al. 2006) was employed. This culture system is designed to provide a better understanding of the skeletal physiology as it maintains the tissue in its 3D structure in a near to physiological environment through the constant perfusion of nutrients and oxygen throughout the tissue (Jones et al. 2003). Furthermore, this system allows the bone cells to interact with each other as well as with the extracellular matrix while the controlled application of mechanical loading or the administration of hormones and growth factors might enable the study of bone remodelling in normal and pathological bone in a near to natural environment.

This pilot study has investigated the effects of "physiological" levels of mechanical loading on the maintenance of the osteocytic population in human bone in the presence or absence of 17β-estradiol following 27 days in culture in the Zetos ^{TM} bioreactor.

6.2 Materials and Methods

Unless indicated otherwise, all reagents were purchased from Sigma, UK. Preparation and culture of bone tissue was performed in a sterilised environment using sterile equipment.

6.2.1 Zetos™ description

The Zetos™ bioreactor consists of two parts; a set of diffusion chambers in which cancellous bone cores are placed and supplied with media by the use of a perfusion system, and a computer-controlled loading device used to apply daily physiological mechanical stimulation to individual cores (**Figure 6.1**), (Mann et al. 2006).

Bone cores are housed within the diffusion chambers where they are immersed in growth media and subjected to mechanical loading. Chambers contain a loading piston at the top plate which transmits the loading force to the bone core while the bottom plate is open in order to measure bone deformation at the bottom of the plate. The media is pumped continuously around the system by the use of a perfusion pump which supplies media to multiple chambers simultaneously. The loading device is controlled by a computer programme and applies a defined load to the bone cores (Jones et al. 2003).

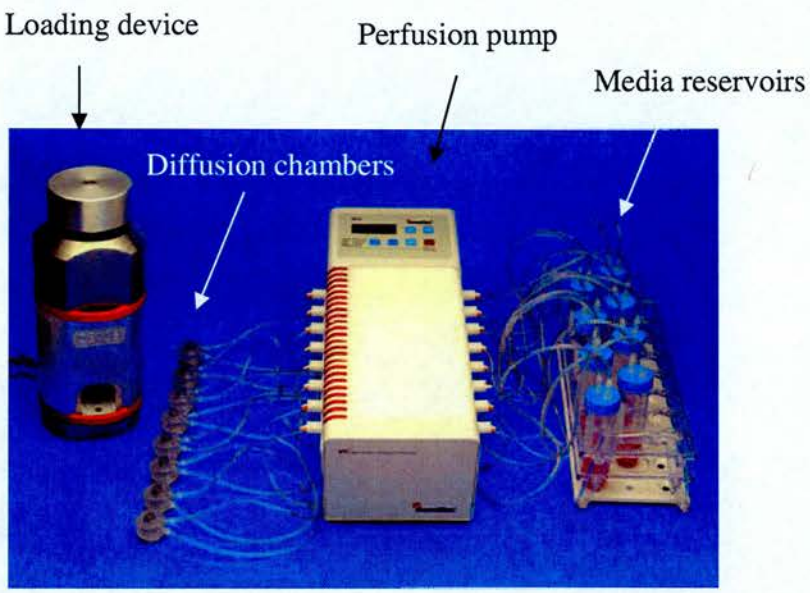


Figure 6.1. The Zetos™ system. The Zetos™ bioreactor consists of a loading device controlled by a computer and diffusion chambers perfused with media through individual reservoirs with the aid of a peristaltic pump.

6.2.2 Culture of bone tissue in the Zetos™ system

6.2.2.1 Preparation of human biopsies for culture

Human femoral head tissue was obtained from a 65 year-old male undergoing total hip arthroplasty with written informed consent and with the approval of the local ethical committee. The samples were processed within 2 hours following the surgery and were cut into 8 mm slices (**Figure 6.2**) in thickness using an Exackt 310 diamond-coated band saw. During the cutting process, the femoral head was continuously rinsed with sterile PBS at 4 °C in order to minimise heat-induced damage to the tissue and to prevent the bone from drying out (Mann et al. 2006). Cylindrical bone cores at 10mm in diameter were drilled from each of these 8 mm-thick slices (**Figure 6.2**) using a diamond-coated hollow drill (made in house), while their height was adjusted to 5mm (± 2μm) with the Exackt 310 diamond coated band saw. The bone cores were then thoroughly washed three times for 10 min in Hank's Balanced Salt Solution (HBSS) at 37°C, and subsequently washed for 20 min in HBSS supplemented with the antibiotics Penicillin/Streptomycin (50IU/ml) (Invitrogen, UK), gentamycin (10mg/ml) and the antimycotic amphotericin B (4μg/ml) in order to minimise the risk of contamination during the harvesting procedure. Cores were randomly assigned into four groups; explants that were subjected daily to mechanical stimulation (n=3) (Loaded), explants that were subjected to mechanical stimulation and received treatment with 17β-estradiol (Calbiochem, UK) at the same time (n=2) (Loaded+E_2), explants that were not subjected to mechanical loading but received 17β-estradiol treatment (n=3) (Unloaded+E_2) and explants that were not subjected to mechanical loading nor received 17β-estradiol treatment (n=2) and provided the untreated control of the study (Unloaded) (**Table 6.1**).

6.2.2.2 Controls samples

As described in § 6.2.2.1, core samples that were not subjected to mechanical loading nor received 17β-estradiol treatment (Unloaded) were included in the Zetos ™ chambers in order to provide negative controls for mechanical stimulation or treatment with 17β-estradiol. Besides the untreated controls that were incubated in the Zetos™ bioreactor for 27 days, additional control samples that were not included in the Zetos™ chamber were

also provided in the study. Two bone cores (T0) were frozen immediately in super cooled hexane after the harvesting procedure in order to provide an indication of the state of the tissue at the beginning of the experiment and to act as positive controls for cell viability assays. In addition, three cores were submerged in sterile H_2O overnight following their extraction from the femoral head and then frozen immediately, in order to induce necrosis as an alternative form of death to apoptosis. This technique is known to induce the bursting of the cells (cytolysis) due to the excessive movement of H_2O molecules through the plasma membrane towards the inside of the cells causing them to explode (Niquet et al. 2004). Bone cores samples subjected to this treatment are termed "dead bone samples" throughout this thesis in order to account for the presence of necrotic osteocytes.

6.2.2.3 Maintenance of bone cores in the Zetos ™ system

Individual cores were inserted into specific diffusion chambers and each diffusion chamber was connected to individual media reservoir via a peristaltic pump system allowing continual perfusion (7ml/hour) of the bone tissue with culture media (**Figure 6.1**). Cores were perfused with Dulbecco's Modified Eagle medium (DMEM) (Gibco, UK) containing 10% FCS, 10mM HEPES, 2mM L-glutamine (Gibco, UK), (10μg/ml) L-ascorbic acid-2-phosphate, 5mM β-glycerophosphate disodium hydrate, 0.14mM $NAHCO_3$ and Penicillin (50 IU/ml) Streptomycin (50μg/ml) (Gibco, UK). Cores were maintained within the 37°C environmental chamber for a period of 27 days (**Figure 6.2**) with media being replaced every third day in order to provide adequate supply of nutrients and remove waste products. Throughout the experimental period, the pH of the media in each diffusion chamber was closely monitored (~ pH 7.25) in order to ensure a physiological pH value during the maintenance of the cores in the Zetos™ bioreactor.

6.2.3 Treatments

6.2.3.1 Loading of the samples

In this chapter, mechanical compressive loading was applied to the bone cores for 5 minutes on a daily basis. The applied forces resulted in strains of 3000 μstrain at a frequency of 1 Hz, in a waveform that corresponded to physiological jumping movement, based on preliminary studies in bovine bone (Mann et al. 2004).

6.2.3.2 Treatment with 17β-estradiol

17β-estradiol was prepared from an initial stock of 10mM in 100% ethanol vehicle and was further diluted to 10μM in normal growth medium. Samples then received treatment with 17β-estradiol at 1μl/ml in normal growth medium to give a final concentration of 10nM. Treatment was administered at the beginning of the experiment (day 1) and was supplied fresh every 3 days into the appropriate chambers throughout the experimental period of 27 days.

6.2.3.3 Fluorescent labelling of bone explants

Calcein label is a fluorochrome that chemically binds to calcified tissues and specifically to the calcium ions present on bone-forming surfaces (Frost 1969, Lee et al. 2003). During the experimental period of 27 days, one calcein label (at a concentration of 2 mg/ml) was added to the culture media on day 6 and was re-circulated in the system for approximately 16 hours in order to allow the fluorochrome to fully bind to the matrix. In order to remove the excess staining, all explants were washed with fresh media for 4 hours which was then removed and replaced as normal.

Since in this study only one label was administered on day 6 of the experiment, any bone material present above the label at the end of the experiment was therefore synthesized during the 21 day period, as described in §6.2.7.2.

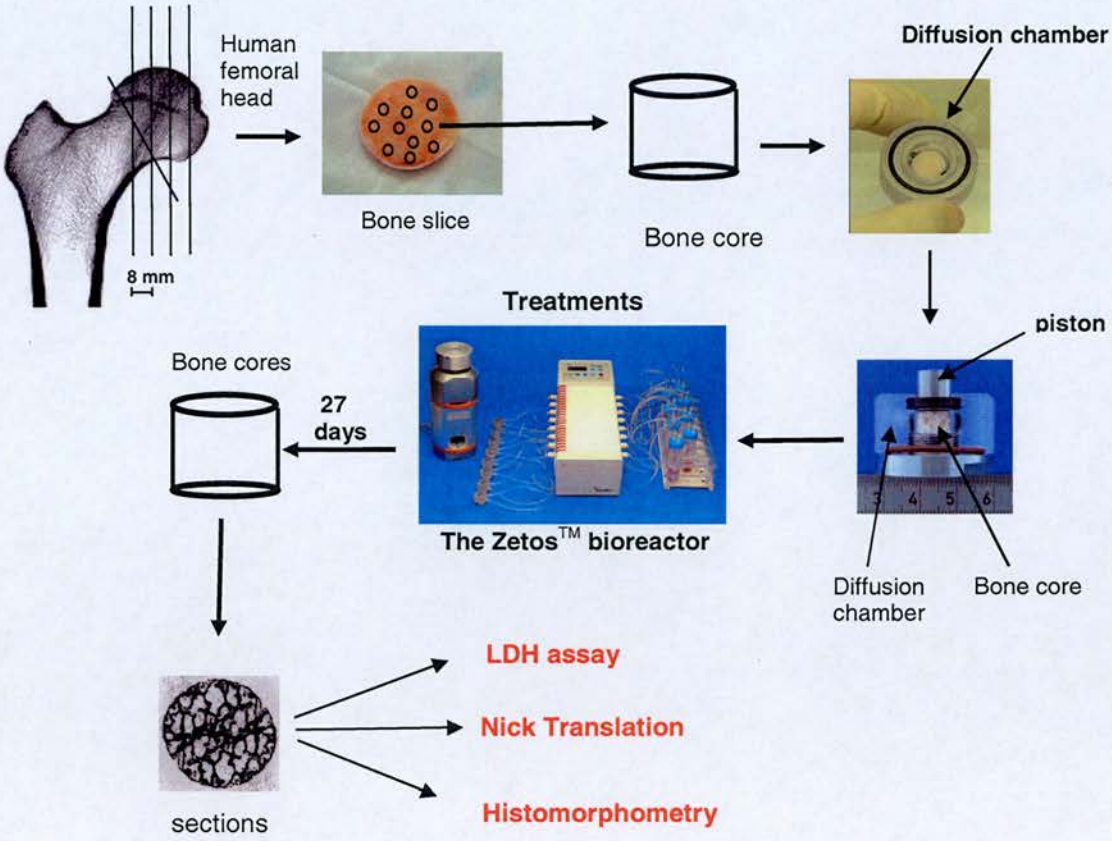


Figure 6.2. Schematic overview of the experimental setup using the 3D bioreactor system (Zetos™). Human trabecular bone cores were drilled from 8 mm-thick bone slices obtained from the human femoral head and housed within the diffusion chambers. Cores were randomly assigned into four different treatment groups (§6.2.2.1) and maintained in the Zetos™ bioreactor for a period of 27 days. Following that period, cores were frozen and sections were collected from each core (see §6.2.4) for subsequent analysis for osteocyte viability, apoptosis and histomorphometric indices.

6.2.4 Sample processing

After 27 days in the bioreactor, bone cores were immersed in 5 % polyvinyl alcohol and snap frozen in hexane prior to storage at -70 ^{0}C. Transverse cryostat sections of 7μm in thickness were cut from the chilled material using an ultra pure tape for sections (Taab Laboratories, UK) and transferred to Superfrost slides (Western Laboratory Services, UK) for further analysis. The use of the ultra pure tape allowed better adhesion of the bone sections including the bone marrow onto the slides. In order to broadly identify appropriate regions for subsequent analysis of osteocyte viability, apoptosis and osteogenic activity, sections were collected from distances 0-0.5mm (Zone 1) and 1-2.5mm (Zone 2) from the upper surface of the bone cores as outlined in **Table 6.1**, and **Figure 6.3**.

Treatments	No of cores /treatment	No of sections /Zone	Total no of sections/ Zone/ treatment	No of fields/ section	Total no of fields /Zone /treatment
T0	2	6	12	6	72
Unloaded	2	6	12	6	72
Unloaded +E_2	3	6	18	6	108
Loaded	3	6	18	6	108
Loaded +E_2	2	6	12	6	72
Dead bone	3	6	18	6	108

Table 6.1. Outline of sections collected and fields analysed from each Zone. Six sections were collected from each Zone per core and six fields of view were analysed for each section, for each of the outcome measures (§6.2.5-6.2.7).

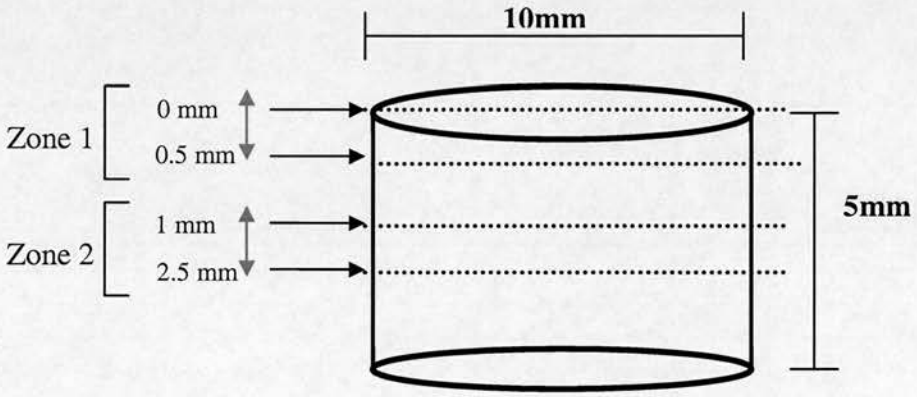


Figure 6.3. A schematic representation of a bone core. Bone cores are cylindrical in shape, 5 mm in height and 10 mm in diameter. The arrows point to the distances between which cryostat sections were collected in order to carry out subsequent analysis. Sections obtained from distances 0-0.5mm were grouped into the Zone 1 sections, while sections between distances 1- 2.5mm comprised the Zone 2 sections.

6.2.5 Cell viability assessment in situ

Osteocytes that were viable at the time of sampling were identified in cryostat sections by means of their lactate dehydrogenase (LDH) activity as described in §3.2.3. The number of LDH positive osteocytes, representing the viable osteocytes, was expressed over the bone area (mm^2) which was measured using the bone histomorphometry software BIOQUANT OSTEO (Bioquant Image Analysis Corporation, USA). LDH staining was performed on three consecutive sections taken at each specified distance through each of the bone cores and six image fields were analysed per section.

6.2.6 In situ analysis of osteocyte apoptosis using Nick Translation

The percentage of osteocytes demonstrating fragmented DNA and therefore considered to be apoptotic, was investigated using in situ DNA nick translation technique (Noble et al. 1997), as previously described in § 3.2.2. Counterstaining was performed using DAPI, at 2.5ng/ml in order to enable visualisation of the osteocyte number in each section. Sections were then visualised by fluorescence microscopy in order to determine the ratio of the apoptotic osteocytes (FITC positive) over the total number of osteocytes (DAPI stained osteocytes). NT technique was employed on three consecutive sections per biopsy and a minimum of eight fields of view were analysed per section taken at each specified distance through the bone cores covering in principle more than 70% of the total bone area at 20x magnification.

6.2.7 Histomorphometry

Bone histomorphometry provides detailed information on bone structure and remodelling at a specific location within the biopsy, whereas other approaches, such as the use of biochemical markers of bone turnover, allow the measurement of histomorphometric indices at the whole skeleton (Chavassieux and Delmas 2006). Histomorphometric indices are categorised into the primary measurements such as measurements of bone volume (BV), surface (BS), distance between points or lines and number (Parfitt 1987) and the derived (from the primary) measurements some of which include the mineralising surface (MS), mineral apposition rate (MAR) and Bone Formation Rate (BFR/BS).

Using fluorescent microscopy, the fluorescent label and the bone surfaces were traced on the sections from the samples maintained in the Zetos^{TM} system and qualitative analysis of the histomorphometric parameters within the tissue sections from all explants was carried out using the specific bone histomorphometry software BIOQUANT OSTEO (Bioquant Image Analysis Corporation, USA). All histomorphometric measurements, analysed in this chapter, are described according to the standard nomenclature system established by the American Society of Bone and Mineral Research (ASBMR) nomenclature committee (Parfitt 1987).

The sections used for histomorphometric analysis were collected from Zones 1 and 2 of each of the bone cores. Three consecutive un-decalcified 7μm thick sections were fixed in 4 % paraformaldehyde (PFA) for 10 minutes, washed in PBS three times and mounted in fluorescent mounting medium (DAKO). Eight random fields from each section obtained from each distance (24 fields in total obtained for each treatment group) were taken at a 20x magnification lens using fluorescent microscopy and analysed using the BIQUANT OSTEO software.

6.2.7.1 Primary measurements

Primary measurement of bone surface (BS) was performed on decalcified sections on a minimum of eight fields obtained from three sections obtained at each of the distances using the formula:

BS (mm) = Total Bone Surface - Artificial edges

6.2.7.2 Derived measurements

Mineralising surface (MS) describes the bone surface (BS) that exhibits both single (sL) and double-labelled (dL) surfaces. Single-labelled surfaces refer to the presence of the calcein label localised at the bone edge while the presence of a second or third label would be localised either on or further away form the edge of the bone. Although this study was limited by the administration of only one label on day 6 of the experiment, interesting results were obtained. In more detail, any bone material existing above the label at the end of the experiment was synthesized during the 21 day period and therefore the edge of the bone on day 27 was considered as a second pseudo label by the histomorphometry software.

The measurement includes the pseudo double plus half of the single labels in order to provide a more accurate explanation of the actively mineralising surface. The Mineralising surface formula employed in this study was:

MS / BS (%) = pdLS + (0.5 x sLS) / BS

where pdLS is the pseudo double-labelled surfaces and sLS is the single-labelled surfaces

The Mineral Apposition Rate (MAR) was estimated by the mean distance between the two calcein labels (first label and pseudolabel) (interlabel thickness) divided by the time period between the administration of the first label and the final day of the experiment (interlabel time-21 days) and represents the rate of progression of the mineralization front (Parfitt et al. 1987).

Measurements were taken at the midpoint of each label at approximately four equidistant points along the pseudo double-labelled surface.

MAR (μm/day) = Ir.L.Th / Ir.L.t

Where Ir.L.Th is the interlabel thickness and Ir.L.t is the interlabel time.

In addition to the primary measurements described above; Trabecular Bone Formation Rate (BFR) was also studied in relation to the bone surface (BFR/BS) obtained by multiplying the MAR by the total mineralising surface (the sum of the pseudo double-labelled surface and half of the single-labelled surface). It expresses the rate of bone formation per unit of bone surface and is calculated as:

BFR/ BS (μm/day) =MAR x MS/BS

6.2.8 Statistical analysis

All data analyses were performed using the statistical software package SPSS for Windows 11.5. All data were checked for normality. In cases where the randomly selected sample data were shown to have a normal (Gaussian) distribution, parametric statistical tests such as the two-tailed Analysis of Variance (ANOVA) followed by the Tukey-Kramer post hoc test (when the significance of the ANOVA was $p<0.05$) were performed in order to determine statistical significance between the treatment groups. For percentages or proportions which were not found to be normally distributed, the square root of each proportion was transformed into its arcsine, which allowed the distribution of the data to be nearly normal enabling the use of parametric tests. Results are presented as means ± SEM. $p< 0.05$.

6.3 Results

6.3.1 Bone Formation Rate (BFR)

All bone cores introduced in the Zetos^{TM} system received calcein label at day 6. A second label was considered to be the bone edge at day 27 of the experiment, which enabled the measurement of bone formation indices in response to various treatments (see § 6.2.3.3, § 6.2.7.2).

As shown in Figure 6.4A, no significant differences in the BFR/BS were observed in response to the different treatments in Zone 1 (**Figure 6.4A**). However, in Zone 2 of the biopsy perimeter, application of mechanical stimulation in the absence of E_2 supplementation significantly increased the BFR/BS compared to the unloaded samples ($p=0.04$), (**Figure 6.4B, Figure 6.5**).

Administration of 17β-estradiol alone to the unloaded samples also induced a significant increase in the BFR/BS compared to the unloaded samples alone in Zone 2 of the cores ($p= 0.001$), (**Figure 6.4B, Figure 6.5**). However, application of mechanical loading in the presence of 17β-estradiol significantly suppressed the increase in bone formation indices induced by 17β-estradiol alone in Zone 2 ($p=0.006$), (**Figure 6.4B**). As shown in Figure 6.4C, no significant differences were identified in the BFR/BS between Zone 1 and Zone 2 in response to individual treatments ($p>0.05$), **(Figure 6.4C**).

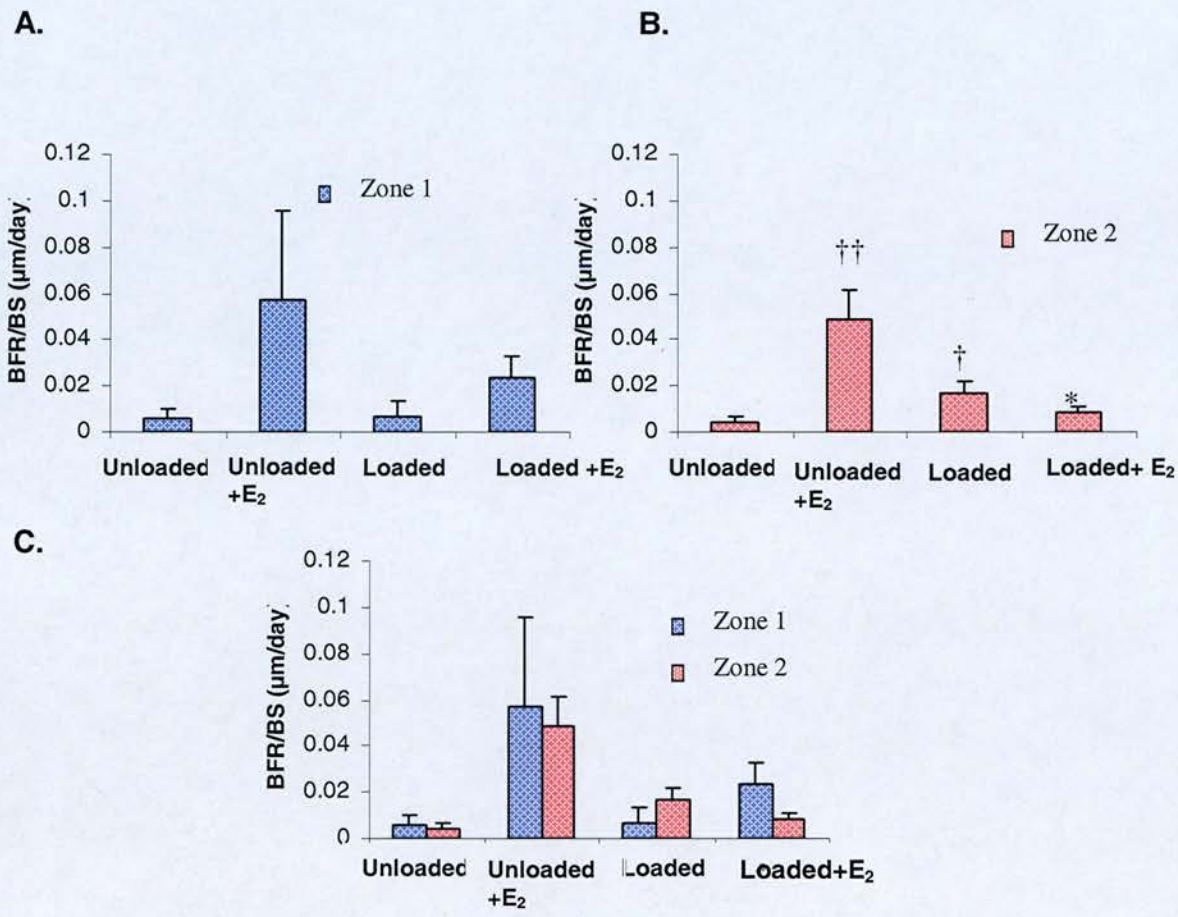


Figure 6.4. The effects of mechanical loading and/or 17β-estradiol administration on the Bone Formation Rate (BFR/BS) after 27 days in the Zetos™ system. Sections were analysed for indices of bone formation using the specific bone histomorphometry software BIOQUANT OSTEO (USA). **A.** There were no differences observed in the BFR/BS measurements between different treatments in Zone 1. **B.** Application of mechanical stimulation increased the rate of bone formation in the sections obtained from Zone 2 compared to the unloaded samples. In addition, administration of 17β-estradiol in the absence of loading increased the BFR/BS compared to all other treatments in Zone 2. Results are expressed as the mean rate of bone formation per unit of bone surface. **C.** There are no differences in the BFR/BS between Zone 1 and Zone 2 in response to individual treatments ± S.E. ††=$p<0.001$, †=$p<0.01$ compared to the unloaded cores in Zone 2, * = $p<0.05$, compared to the E_2-treated bone cores in Zone 2.

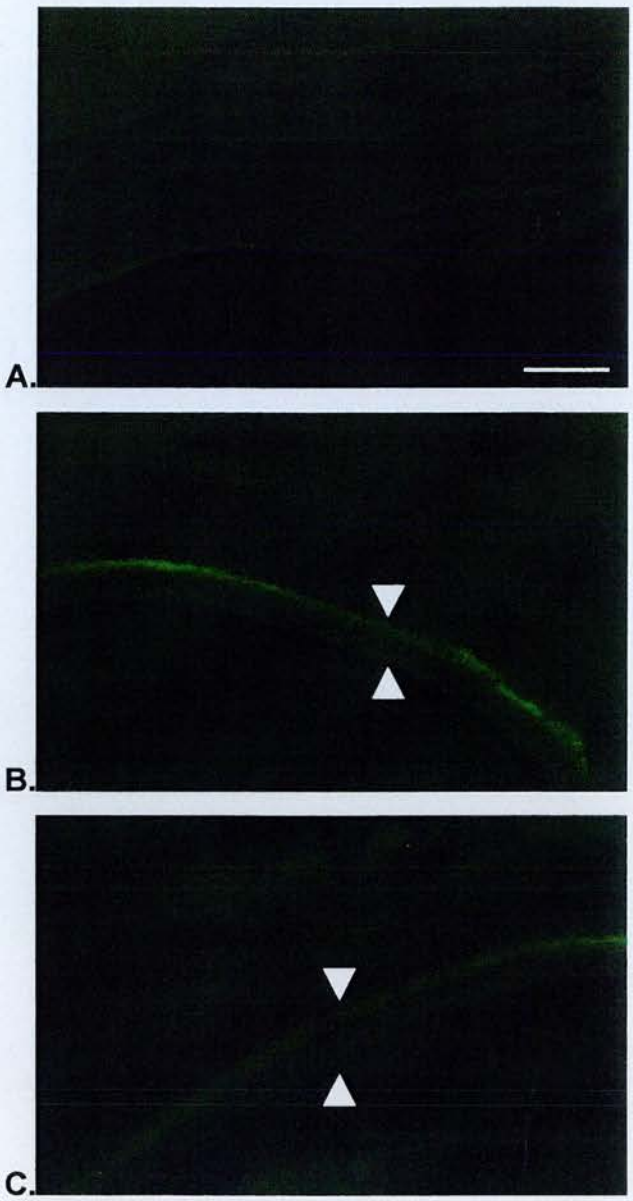


Figure 6.5. Calcein label and Bone Formation Rate. Calcein label was administered on day 6 of the 27 day experiment. Bone overlying the calcein label was measured using the histomorphometry software. Representative images of **A.** the unloaded sample showing no label, **B.** mechanically stimulated sample and **C.** 17β-estradiol- treated sample showing calcein label and newly formed bone (arrowheads). Bar = 100 μm.

6.3.2 Mineral Apposition Rate (MAR)

Sections were also analysed for the Mineral Apposition Rate (MAR), as described in methods. As shown in Figure 6.6A, no significant differences were observed in the MAR between the different treatments in Zone 1 (Figure 6.6A).

However, in Zone 2, application of mechanical loading increased the MAR compared to the unloaded samples ($p=0.04$) (**Figure 6.6B**). In addition, the MAR was also increased in the unloaded samples that received 17β-estradiol and was significantly higher compared both to the unloaded samples alone and to the samples that received both mechanical loading and 17β-estradiol in Zone 2 ($p=0.038$, and $p=0.0004$, respectively), (**Figure 6.6B**). As shown in Figure 6.6C, there are no significant differences in the MAR between Zone 1 and Zone 2 in response to individual treatments ($p>0.05$), **(Figure 6.6C**).

6.3.3 Percentage of pseudo -double labelled surface (% pdL/BS)

Sections were further analysed for the percentage of pseudo double-labelled surface (% pdL/BS). Application of mechanical stimulation for 27 days in the Zetos^{TM} bioreactor significantly increased the percentage of the pseudo doubled-labelled surface compared to the unloaded samples in Zone 2 ($p=0.01$), (**Figure 6.7B**), but not in Zone 1 (**Figure 6.7A)**. The presence of 17β-estradiol in the unloaded cores, as shown in Figure 6.7, also induced a significant increase in the % pdL/BS compared to the unloaded samples alone in Zone 2 ($p=0.0007$) sections as well as in Zone 1 sections ($p=0.019$) (**Figure 6.7A** and **Figure 6.7B**). Furthermore, it was observed that the administration of 17β-estradiol to bone cores induced a similar increase in the % pdL/BS both in the presence and the absence of mechanical loading. However, there was no significant increase in the % pdL/BS observed in samples that received both loading and 17β-estradiol treatment compared to the unloaded samples alone in Zone 2 ($p>0.05$), (Figure 6.7B).

As shown in Figure 6.7C, there are no significant differences in the % pdL/BS between Zone 1 and Zone 2 in individual treatments ($p>0.05$), **(Figure 6.7C**).

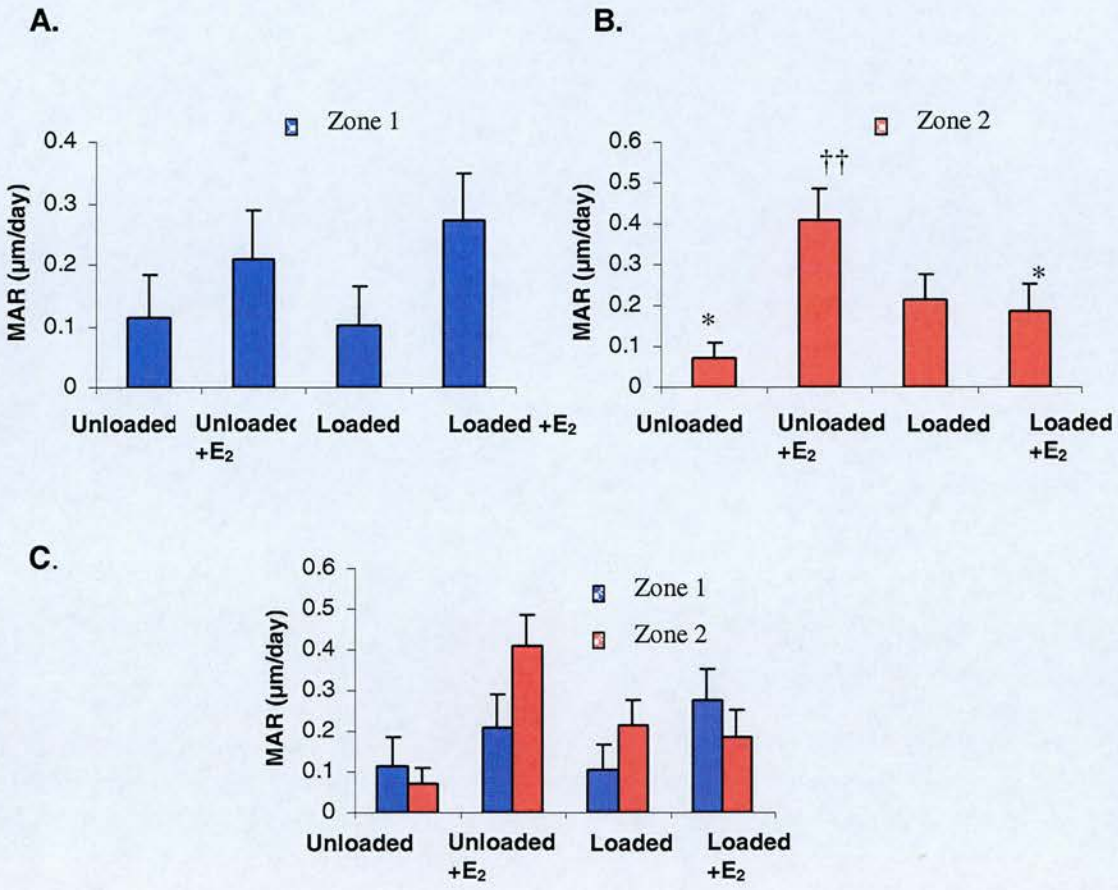


Figure 6.6. The effects of mechanical loading and/or 17β-estradiol administration on the Mineral Apposition Rate (MAR) after 27 days in the Zetos™ system. Sections were analysed for indices of mineral apposition using the specific bone histomorphometry software BIOQUANT OSTEO (USA). **A.** There are no differences in the MAR between different treatments in Zone 1. **B.** Administration of 17β-estradiol to the unloaded samples increased the MAR both compared to the unloaded samples alone and to the loaded samples treated with 17β-estradiol in Zone 2. Results are expressed as the mean mineral apposition rate. **C.** There are no differences in the MAR between Zone 1 and Zone 2 in response to individual treatments. ± S.E. †† = $p < 0.001$, † = $p<0.05$ compared to unloaded cores in Zone 2, * = $p < 0.05$, compared to E_2-treated bone cores in Zone 2.

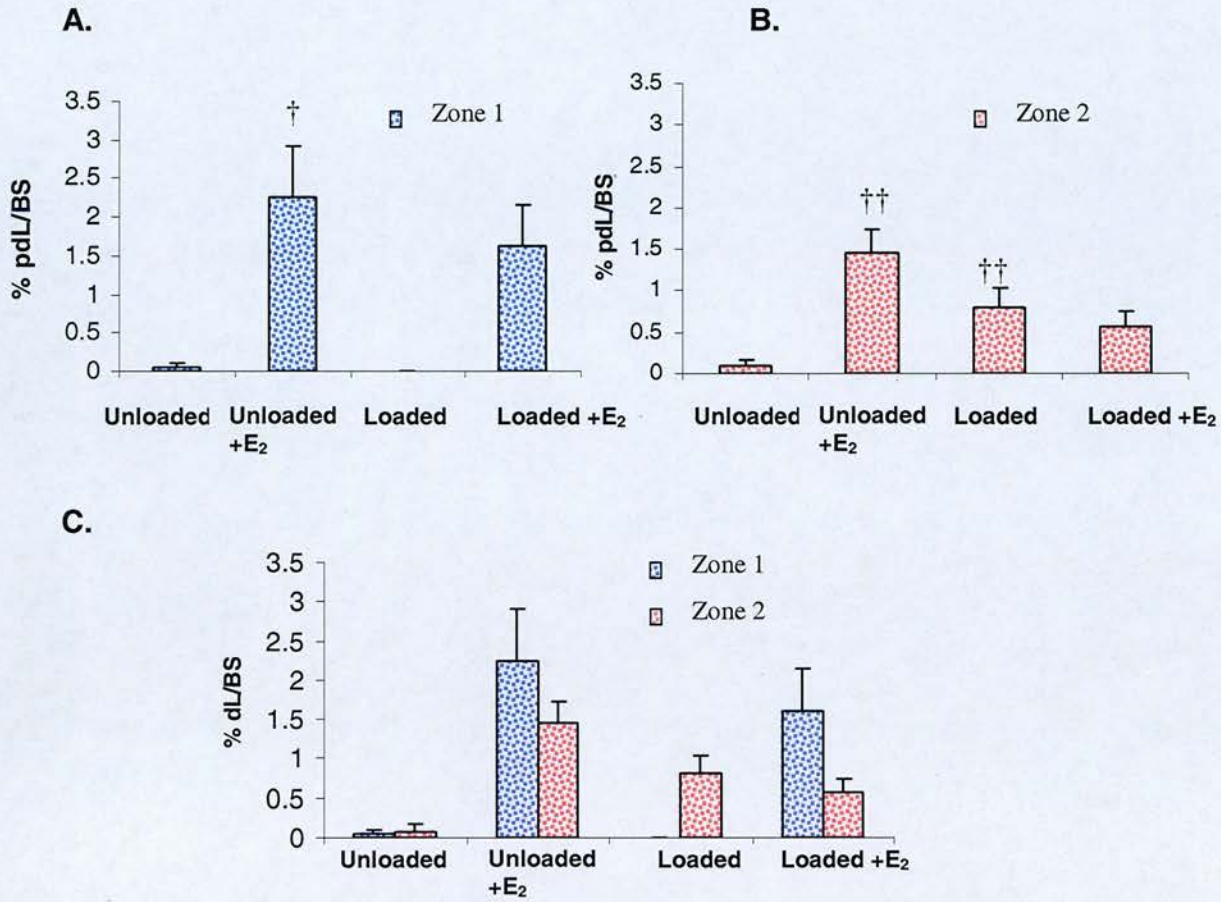


Figure 6.7. The effects of 17β-estradiol administration and/or loading application on the % pdL/BS after 27 days in the Zetos™ system. Surfaces were analysed for indices of pseudo double calcein labels using the specific bone histomorphometry software BIOQUANT OSTEO (USA). **A-B.** Administration of 17β-estradiol to the unloaded samples increased the % pdL/BS compared to the unloaded samples both in Zone 1 and Zone 2. **B.** Application of mechanical loading increased the % pdL/BS compared to the unloaded samples in Zone 2. **C.** There are no differences in the % pdL/BS between Zone 1 and Zone 2 in response to individual treatments. Results are expressed as the mean % pdL/BS. ± S.E. †† = $p < 0.001$, † = $p < 0.05$ compared to the unloaded cores in A and B.

6.3.4 Osteocyte viability at day 0 post-harvest from the human femoral head.

In order to provide positive controls for the viability of osteocytes prior to the introduction of the cores to the Zetos^{TM} system, two samples were frozen immediately following the harvesting procedure, as described in methods, and were termed time zero (T0) controls. Cryostat sections obtained from Zone 1 and Zone 2 were analysed for LDH activity per mm^2 of bone tissue, as described in §6.2.4 and §6.2.5. **Figure 6.8** shows that osteocyte viability in Zone 2 was significantly higher compared to Zone 1 in the T0 controls ($p=0.0002$).

6.3.5 Osteocyte apoptosis at day 0 post-harvest from the human femoral head

T0 cores were also examined for indices of osteocyte apoptotic death, which was estimated as the proportion of osteocytes displaying DNA fragmentation in situ against the total number of osteocytes per field of view as described in methods, at sections obtained from either Zone 1 or Zone 2. As demonstrated in **Figure 6.9**, the percentages of apoptotic osteocytes were similar between Zone 1 and Zone 2 in the T0 cores ($p>0.05$).

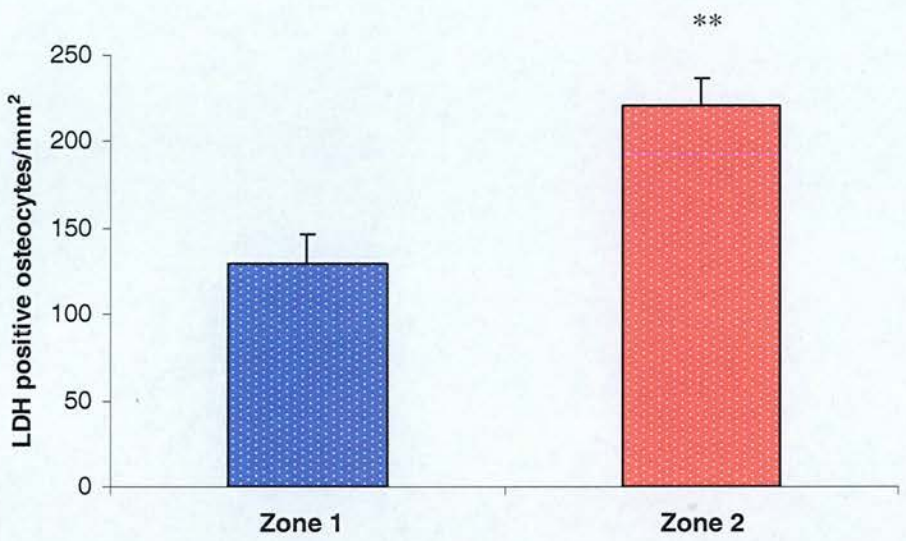


Figure 6.8. The density of LDH positive osteocytes at 0 days post-harvest. T0 cores were harvested and frozen prior to entry in the Zetos™ system and sections collected from Zone 1 and Zone 2 were analysed for the presence of LDH activity in osteocytes. In sections obtained from Zone 2 the viability of osteocytes per mm^2 was higher compared to sections obtained from Zone 1. Results are expressed as the average number of the LDH positive osteocytes per area (mm^2) in sections derived from Zone 1 and Zone 2 ± *S.E.* ** $p<0.001$ compared to Zone 1.

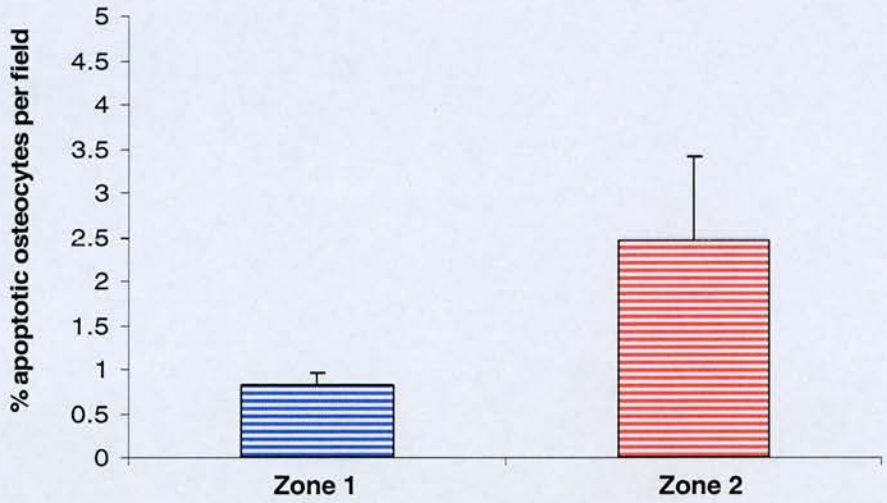


Figure 6.9. The percentage of osteocytes displaying evidence of DNA fragmentation in situ in the T0 controls. T0 bone cores were frozen prior to the entry in the Zetos^{TM} system and sections obtained from both Zone1 and Zone 2 were examined for the presence of fragmented DNA material using an in situ nick translation technique. The percentage of apoptotic osteocytes in the T0 controls was similar between Zone 1 and Zone 2. Results are expressed as the mean percentage of osteocytes displaying positive staining for fragmented DNA over the total number of osteocytes per field of view. ± S.E.

6.3.6 Negative control for osteocyte viability

In order to provide controls for low LDH activity/mm^2 three cores were incubated in H_2O overnight following their extraction from the femoral head in order to induce necrosis to osteocytes as an alternative form of death to apoptosis (Niquet J et al., 2004). Bone cores samples subjected to this treatment are termed "dead bone samples" throughout this thesis, to account for the presence of necrotic osteocytes. Sections were analysed for osteocyte viability using the LDH staining method (see §6.2.5). **Figure 6.10** shows that the number of viable osteocytes in the dead bone samples was significantly lower compared to the number of viable osteocytes in the T0 samples, analysed either in Zone 1 or Zone 2 sections ($p<0.0001$).

As shown in **Figure 6.10**, bone cores that were subjected to this treatment, contained very few or no viable osteocytes and therefore the nick translation technique for detection of any apoptotic indices was not employed in these samples.

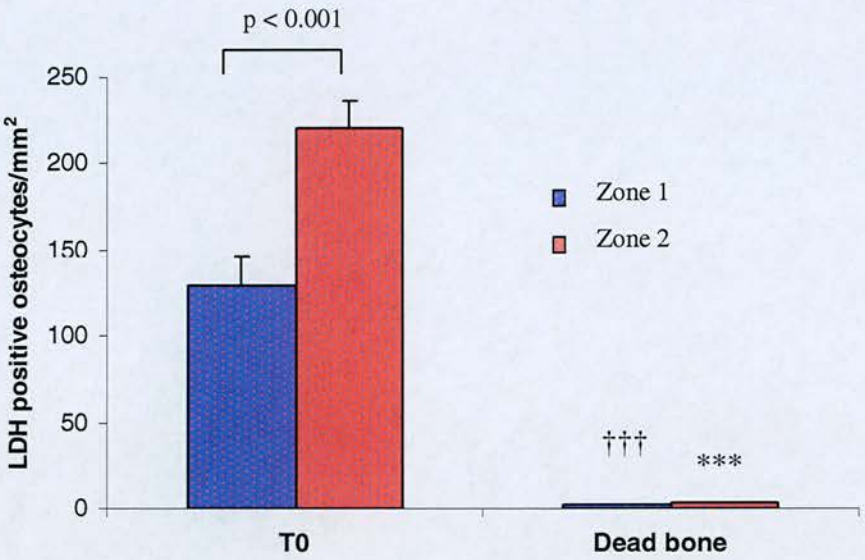


Figure 6.10. The density of LDH positive osteocytes in the dead bone samples. Cryostat sections collected from Zone1 and Zone 2 of the dead bone cores were analysed for the presence of LDH activity in osteocytes. The number of viable osteocytes in the dead bone samples was found to be lower compared to the T0 samples in both ■= Zone 1 and ■= Zone 2. Results are expressed as the mean number of LDH positive osteocytes per area (mm^2) of bone ± S.E. †††= $p<0.0001$, compared to the T0 in Zone 1, *** = $p<0.0001$ compared to the T0 in Zone 2.

6.3.6 The effect of mechanical loading on osteocyte viability

Following their extraction from the femoral head, three bone explants were introduced into the Zetos^{TM} bioreactor and subjected to mechanical stimulation on a daily basis for 27 days as described in §6.2.3.1. In addition, two further cores were maintained in the Zetos^{TM} system in the absence of mechanical loading (disuse condition) in order to provide appropriate negative controls for the samples that received loading. After 27 days in culture, both treatment groups were analysed for cell viability using the LDH staining method (see §6.2.5).

The number of viable osteocytes observed in the T0 samples was significantly higher compared to either the loaded or the unloaded samples ($p<0.0001$). However, the viability of osteocytes in samples maintained in the Zetos^{TM} bioreactor in the presence or absence of mechanical stimulation was significantly higher when compared to the dead bone samples ($p<0.001$) both in Zone 1 and Zone 2 sections **(Figure 6.11A-B, Figure 6.12).**

As demonstrated in **Figure 6.11B,** the number of viable osteocytes in Zone 2 was significantly higher following the application of mechanical stimulation to bone cores compared to the unloaded samples ($p<0.009$) after 27 days in the Zetos^{TM} bioreactor. In addition, as shown in **Figure 6.11C**, no significant difference was found in the number of viable osteocytes between sections obtained from Zone 1 and Zone 2 in any of the treatment groups ($p>0.05$).

6.3.7 The effect of mechanical loading on osteocyte apoptosis

Samples that were deprived of mechanical stimulation in the Zetos^{TM} system exhibited increased osteocyte apoptosis after 27 days of unloading, as indicated in Figures 6.13A and 6.13B. Application of mechanical stimulation in the Zetos^{TM} system reduced the proportion of osteocytes that appeared apoptotic compared to the unloaded samples both in Zone 1 ($p< 0.05$), (**Figure 6.13A**) and Zone 2 ($p <0.0001$), (**Figure 6.13B**) after 27 days of treatment. Furthermore, it was observed that the levels of apoptosis in samples that received mechanical stimulation were significantly lower than those observed in samples that did not receive mechanical loading (**Figure 6.13A, Figure 6.13B**).

Comparison between the levels of apoptosis in Zones 1 and 2 revealed significant differences in the unloaded samples so that Zone 2 sections contained a higher number of apoptotic osteocytes compared to Zone 1 ($p<0.001$), (**Figure 6.13C**).

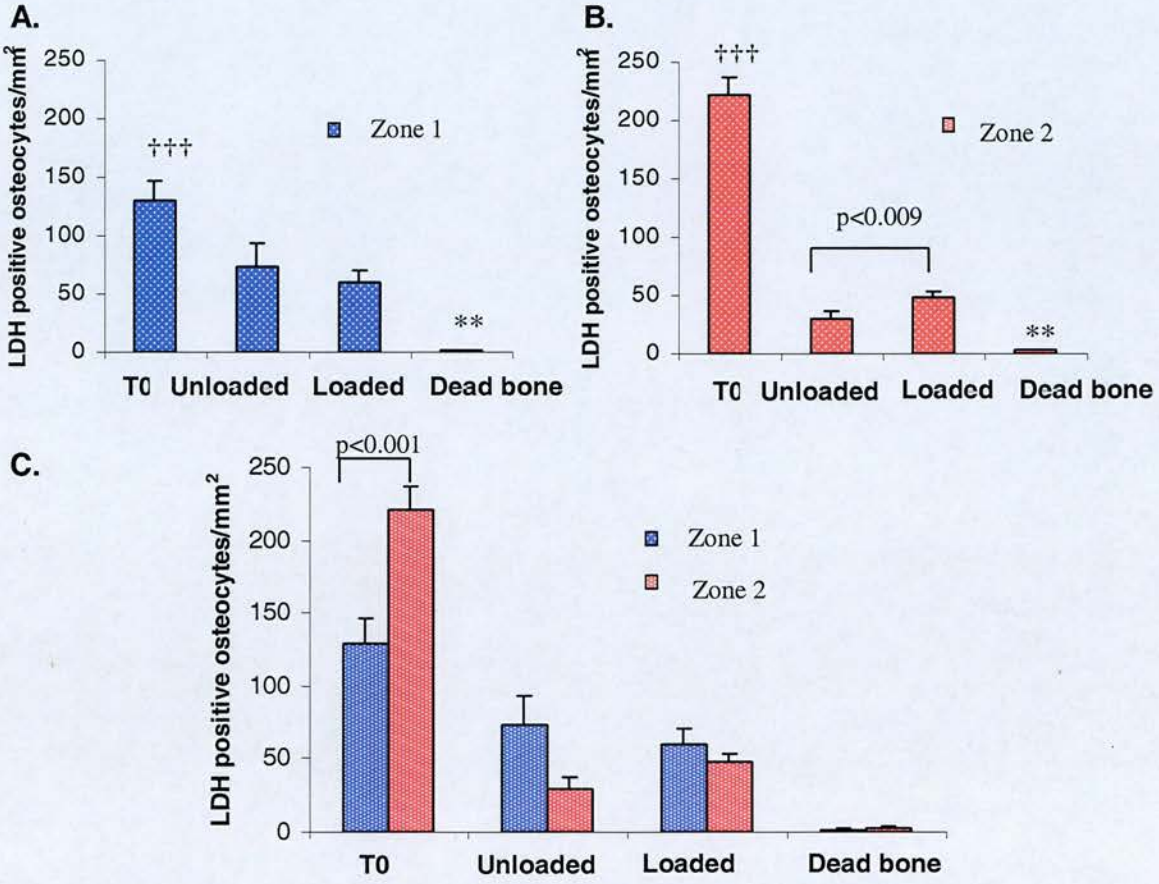


Figure 6.11. Osteocyte viability in the presence or absence of mechanical loading. Bone cores were maintained for 27 days in the Zetos™ system in the presence or absence of mechanical stimulation and examined for LDH activity in osteocytes in both Zone 1 and Zone 2 sections. **A-B.** The number of viable osteocytes in either the loaded or the unloaded samples was lower compared to the T0 samples and higher compared to the dead bone samples both in **A.** Zone 1 and **B**. Zone 2. **B.** Application of mechanical stimulation increased the number of viable osteocytes compared to the unloaded samples in Zone 2. **C.** Besides the T0 control, the density of viable osteocytes was found to be similar between Zones 1 and 2 in all treatments. Results are expressed as the mean number of the LDH positive osteocytes per area (mm^2) of bone ± *S.E.* †††= $p<0.0001$ and **=$p<0.001$ denote significant differences of T0 and necrotic bone respectively to the other treatments.

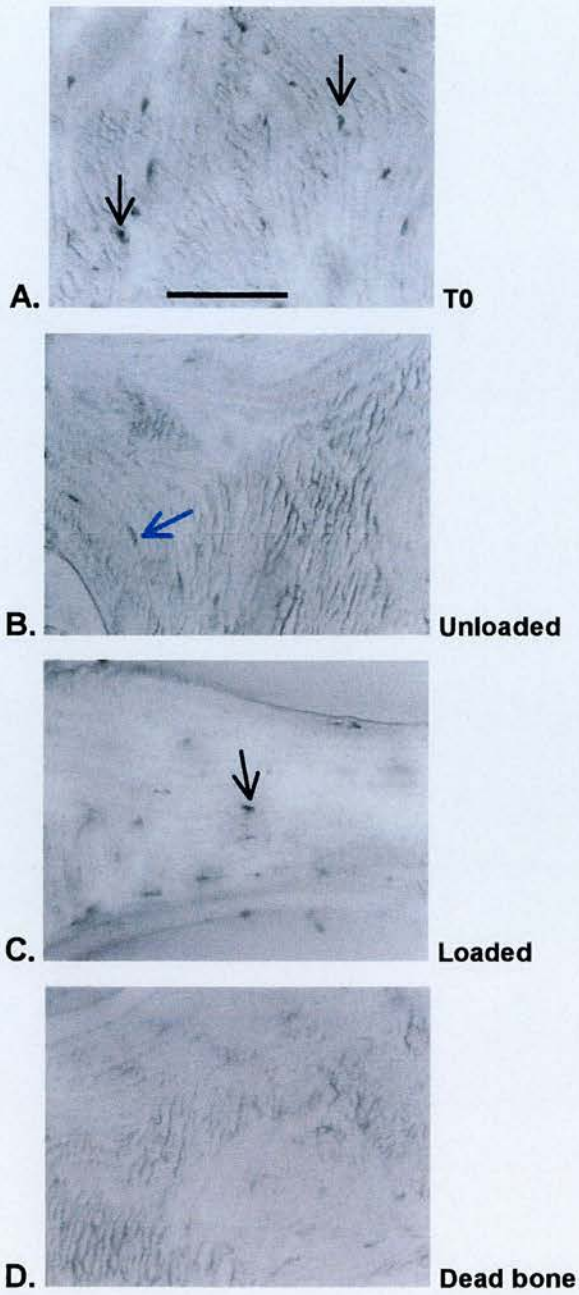


Figure 6.12 Histochemical reaction of LDH enzyme in sections of human bone explants cultured in the Zetos system for 27 days. Osteocyte lacunae showed either strong reaction with LDH indicating viable osteocytes or no reaction indicating empty lacunae or non-viable osteocytes. Representative images from the **A.** T0 control, **B.** unloaded samples, **C.** mechanically stimulated samples and **D.** dead bone samples. Black arrows identify viable LDH-positive osteocytes. Blue arrow points to lacunae which were excluded from measurement (x20). Bar represents 100μm.

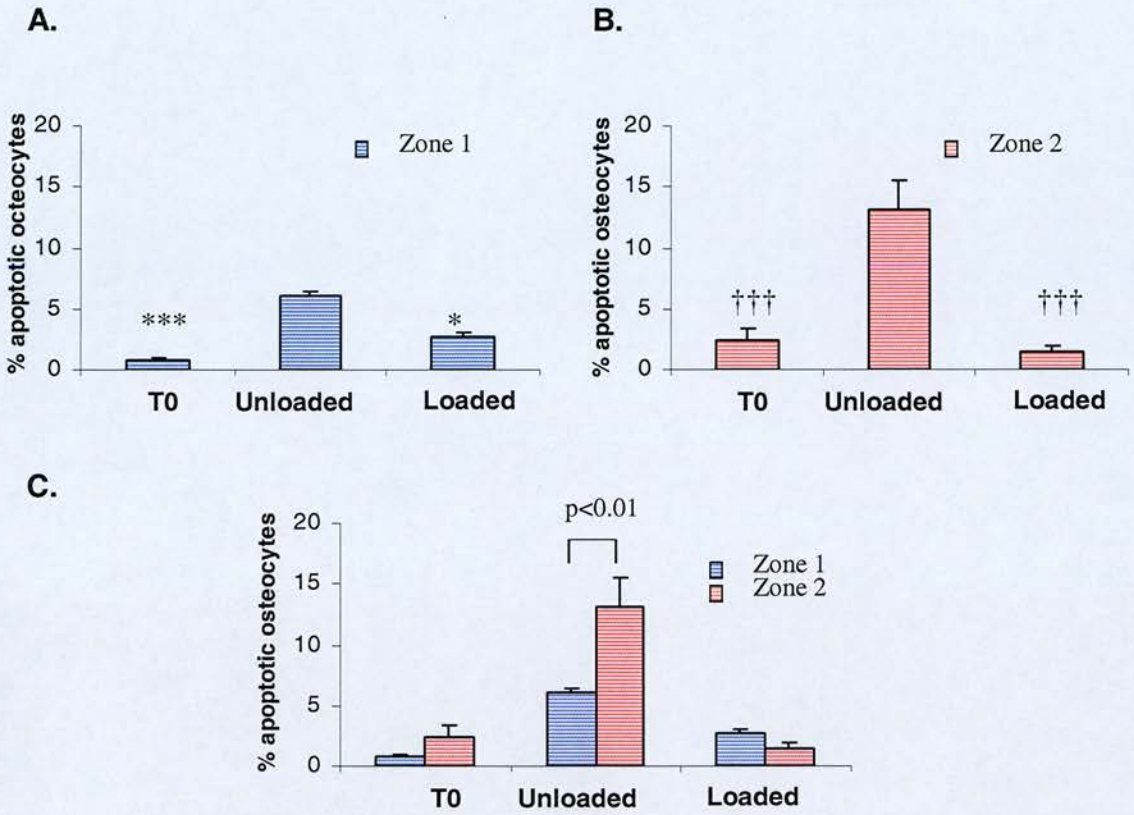


Figure 6.13. The percentage of apoptotic osteocytes displaying evidence of DNA fragmentation in situ in the presence or absence of mechanical loading. Sections collected from both the unloaded and the loaded cores were examined for the presence of fragmented DNA using the in situ nick translation technique. The percentage of apoptotic osteocytes in the unloaded samples was higher compared to either the T0 control or the loaded samples in sections obtained either from **A.** Zone 1 or **B.** Zone 2 of the cores. **C.** Comparison of the levels of apoptosis between Zones 1 and 2 revealed significant differences only in the unloaded samples. Results are expressed as the mean percentage of the osteocytes displaying positive staining for fragmented DNA over their total number. ± S.E. * = $p<0.05$ and *** = $p<0.0001$ denote significance compared to the unloaded samples in A and ††† = $p<0.0001$, denotes significance compared to the unloaded samples in B.

6.3.8 The effect of mechanical loading on osteocyte viability in the presence or absence of 17β-estradiol

Three bone explants received treatment with 17β-estradiol at a concentration of 10^{-8}M supplied fresh every three days for a period of 27 days in the Zetos^{TM} bioreactor in the absence of loading, as described in the method section. Furthermore, two bone cores were subjected to repeated loading cycles and additionally received similar 17β-estradiol treatment. After 27 days, both groups were analysed for cell viability using the LDH activity (see §6.2.5) in order to determine any changes in the number of viable osteocytes following mechanical stimulation in the presence or absence of 17β-estradiol.

The number of viable osteocytes in either the loaded or the unloaded samples in the presence or absence of 17β-estradiol was lower compared to the T0 samples ($p<0.0001$) in sections obtained from both Zone 1 (**Figure 6.14A**) and Zone 2 (**Figure 6.14B**) ($p<0.0001$).

Furthermore, as demonstrated in **Figure 6.14**, the density of viable osteocytes observed in the unloaded samples was not affected following the administration of 17β-estradiol either in Zone 1 (**Figure 6.14A**) or Zone 2 (**Figure 6.14B**). In addition, mechanical stimulation in the presence of 17β-estradiol did not significantly affect the density of viable osteocytes present in the loaded samples both in Zone 1 (**Figure 6.14A**) and Zone 2 (**Figure 6.14B**), ($p>0.05$). However, mechanical strain did not affect osteocyte viability in the absence of 17β-estradiol in Zone 1($p>0.05$) (**Figure 6.14A**).

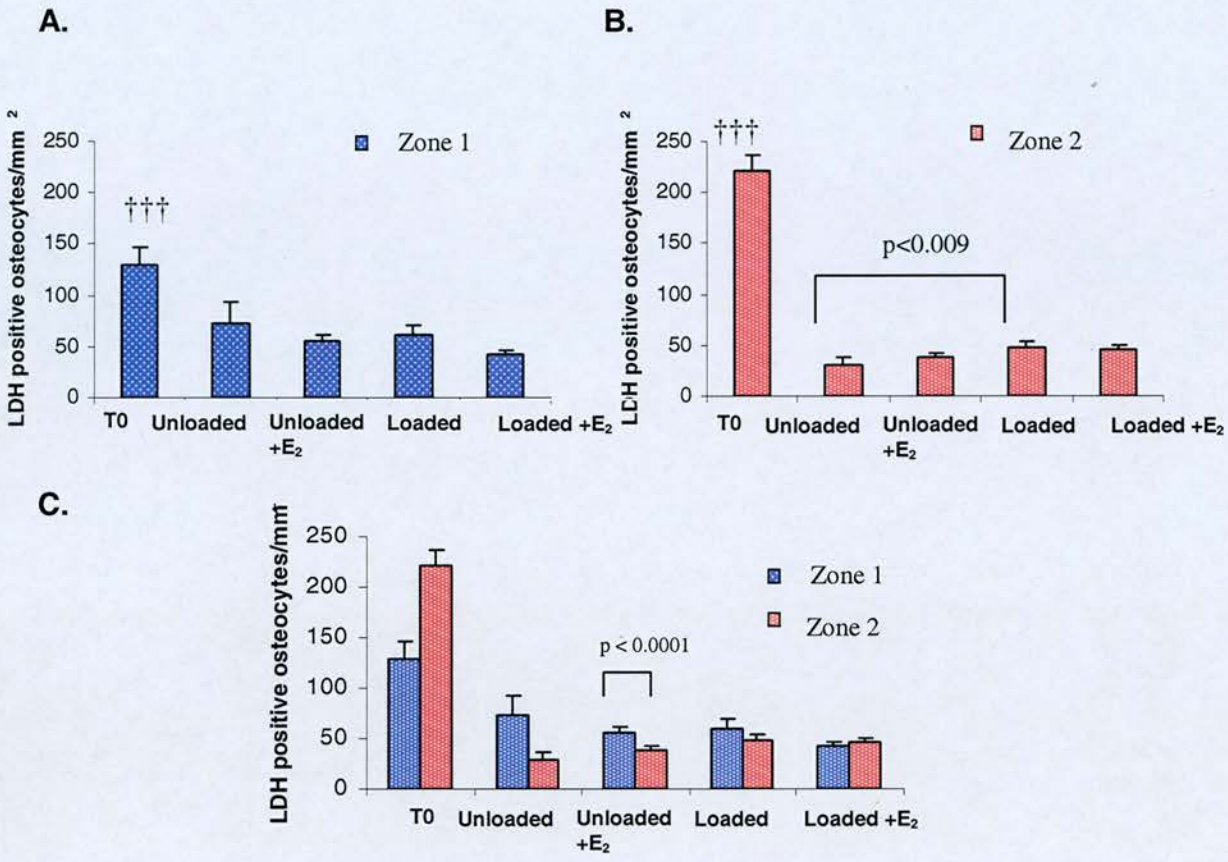


Figure 6.14. The density of LDH positive osteocytes in the presence or absence of mechanical loading and 17β-estradiol. Cores were maintained in the Zetos^{TM} system in the presence or absence of mechanical loading and/or 17β-estradiol for 27 days. Sections collected from Zone 1 and Zone 2 were analysed for the presence of LDH activity in osteocytes. **A-B.** Mechanical stimulation in the presence of 17β-estradiol did not change the density of viable osteocytes induced by loading conditions alone. **C.** Treatment of unloaded bone cores with 17β-estradiol significantly increased osteocyte viability in Zone 1 compared to Zone 2. Results are expressed as the mean number of the LDH positive osteocytes per area (mm^2) ± S.E. †††= $p<0.0001$ denotes significance compared to all the different treatments in Zone 1 (A) and Zone 2 (B).

6.3.9 The effect of mechanical loading on osteocyte apoptosis in the presence or absence 17β-estradiol

Bone cores treated with 17β-estradiol in the presence or absence of mechanical loading were also analysed for indices of osteocyte apoptosis in Zones 1 and 2, as described in methods (Figure 6.16).

Application of mechanical loading in the presence of 17β-estradiol significantly increased osteocyte apoptosis compared to samples subjected to mechanical stimulation alone in Zone 2 (**Figure 6.15B**), ($p=0.001$). Furthermore, administration of both mechanical stimulation and 17β-estradiol to bone cores reduced the apoptosis induced in response to 17β-estradiol treatment alone in Zone 1 (**Figure 6.15A**), ($p=0.008$).

In addition, in samples that received mechanical stimulation in the presence of 17β-estradiol, the levels of osteocyte apoptosis in Zone 2 were higher than in Zone 1 (**Figure 6.15C**), ($p<0.001$). Administration of 17β-estradiol to the unloaded cores did not alter the levels of osteocyte apoptosis induced in response to the unloading conditions alone either in Zone 1 (**Figure 6.15A**) or Zone 2 (**Figure 6.15B**).

Furthermore, the percentage of apoptotic osteocytes appeared higher compared to the T0 controls following the administration of 17β-estradiol either in the presence or absence of mechanical stimulation both in Zone 1 (Figure 6.11A) and Zone 2 (Figure 6.11B), ($p<0.0001$).

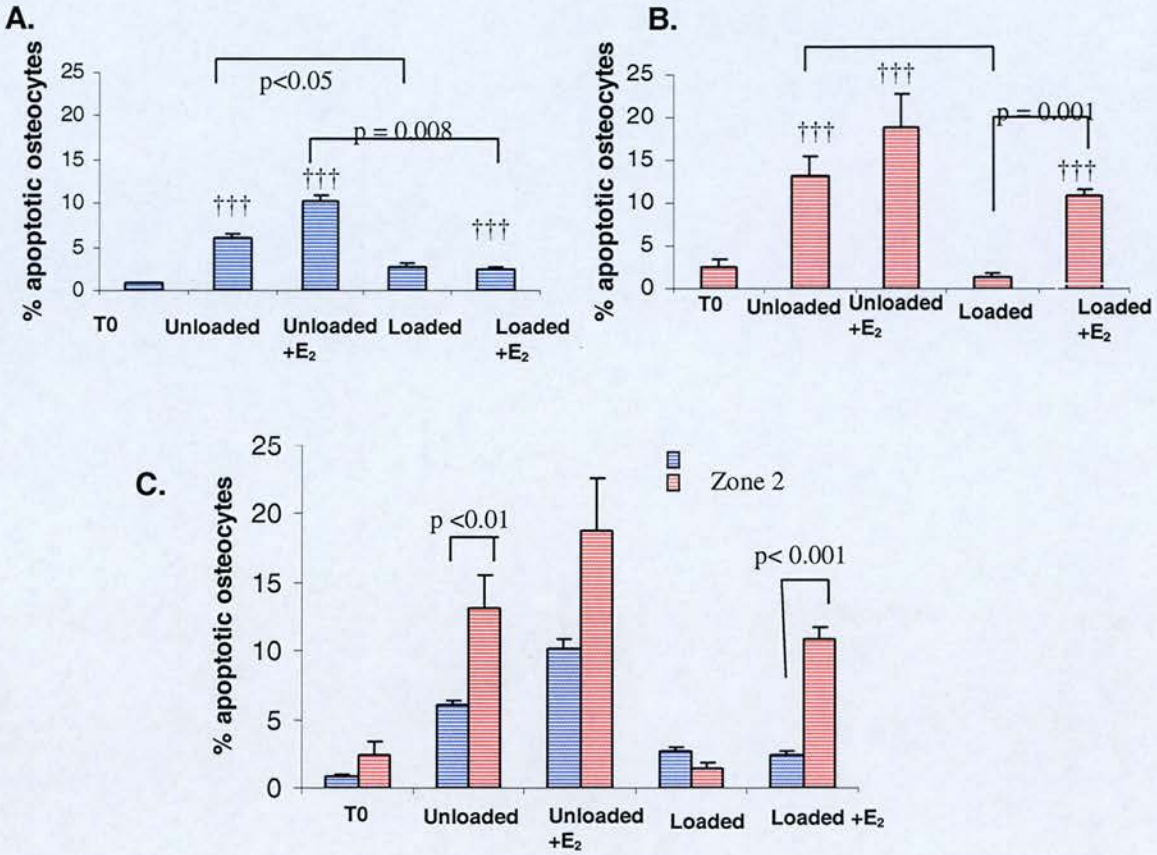


Figure 6.15. In situ evidence of DNA fragmentation in osteocytes subjected to mechanical stimulation in the presence or absence of 17β-estradiol. Sections obtained from Zone 1 and Zone 2 were analysed for osteocyte apoptosis using the in situ nick translation technique. **A-B.** Treatment of bone cores with 17β-estradiol either in the presence or absence of loading increased the percentage of apoptotic osteocytes compared to the T0 controls. Application of loading in the presence of 17β-estradiol reduced osteocyte apoptosis induced in response to **A.** 17β-estradiol alone in Zone 1 and **B.** mechanical stimulation alone in Zone 2. **C.** The percentage of apoptotic osteocytes in samples that received both mechanical loading and 17β-estradiol was higher in Zone 2 compared to Zone 1. Results are expressed as the mean percentage of the osteocytes displaying positive staining for fragmented DNA over their total number. ± S.E. †††= $p<0.0001$ denotes significance compared to the T0 control.

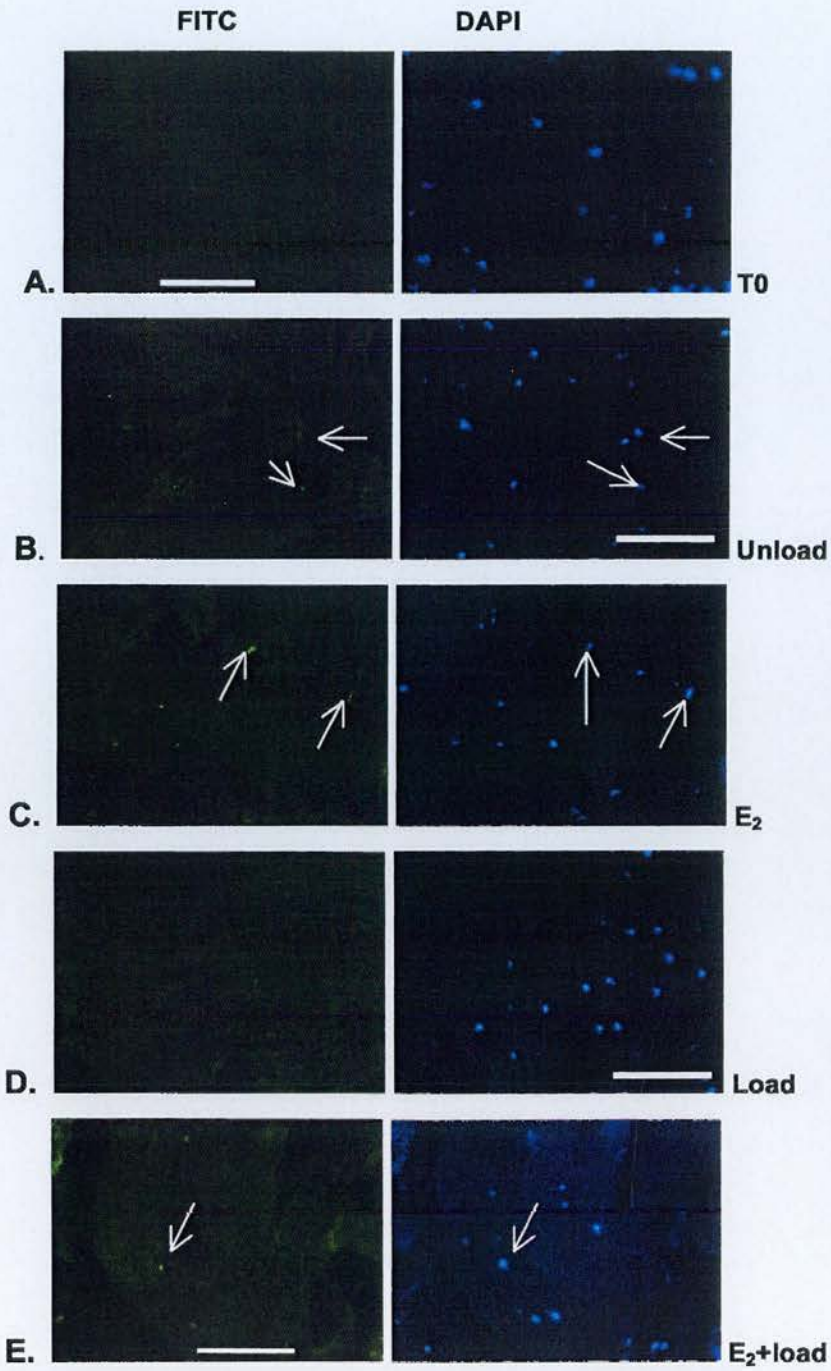


Figure 6.16 In situ evidence of DNA fragmentation in osteocytes subjected to mechanical loading in the presence or absence of 17β-estradiol. The percentage of apoptotic osteocytes was quantified in sections after NT staining (FITC). Representative images of apoptotic osteocytes (arrows) and the corresponding total cell DAPI stain in **A.** T0, **B.** unloaded, **C.** 17β-estradiol-treated, **D.** mechanically stimulated and **E.** 17β-estradiol-treated samples in the presence of mechanical loading. Bar=100 μm.

6.4 Discussion

Data presented in the previous chapters demonstrated a protective effect exerted by 17β-estradiol on the viability of murine osteocytes *in vitro* and rat osteocytes *in vivo*. The study presented in this chapter attempted to investigate the potential effects of 17β-estradiol in the presence or absence of physiological loading on the viability of human osteocytes ex vivo after 27 days in culture using the Zetos^{TM} bioreactor.

A great line of evidence is available to suggest that mechanical loading is able to regulate skeletal function and architecture (Frost 1998, Lanyon 1987, Lanyon 1992). According to the mechanostat theory (Frost 1987), as well as experimental evidence (Mosley et al 1997, Lanyon 1987, Lanyon 1992), application of mechanical loading within the physiological range of strains has been shown to promote anabolic effects to bone, while unloading conditions induced resorption of bone and bone loss. The study presented here investigated possible changes induced in histomorphometric indices in response to unloading conditions or to the application of physiological loading by the Zetos^{TM} bioreactor on human bone core explants.

After 27 days in culture, application of mechanical stimulation in the Zetos^{TM} system increased the rate of bone formation with reference to the bone surface (BFR/BS) compared to the unloading conditions. These findings indicated that the mechanical strains produced in the Zetos^{TM} bioreactor induced an osteogenic response in the bone cores even in the absence of supplementation of known osteogenic factors such as 1, 25 vitamin D3. The BFR/BS induced in response to mechanical loading was estimated to be of the order of 0.02 μm/day and found to be within the lowest range of the BFR/BS reported in the literature for the human iliac crest (Compston et al. 2002). The low values obtained for the BFR/BS variable could be explained by the fact that a pseudo label was used instead of a second fluorescent label in order to calculate dynamic indices of bone turnover. However, to our knowledge there are no histomorphometric estimates available so far regarding the BFR/BS estimates for the human femoral head bone in order to allow comparison with the data presented in this study. The increased BFR/BS observed in this study is in agreement with the mechanostat theory (Frost 1987) as well as with in vivo studies (Chow et al. 1993, Pead et al. 1988, Jagger et al. 1995, Chambers et al. 1993,

Zetos™ ex vivo experimental system has the potential to mimic the response of trabecular bone to physiological strains sensed in vivo.

Furthermore, application of mechanical loading increased the proportion of pseudo double-labelled surfaces (% pdL/BS) as well as the mineral apposition rate (MAR) compared to the unloaded samples after 27 days in culture. These findings might suggest that application of mechanical loading might have induced the conversion of previously quiescent surfaces into active bone forming surfaces (Chow et al. 1993) and the increase in the mineralising activity (productivity) of osteoblasts (Parfitt et al. 1987).

Mechanical loading is known to directly influence the viability of cells that sense mechanical forces in some tissues (Dimmeler et al 1998). Having shown that the application of strains within the physiological range in the Zetos™ bioreactor induced an osteogenic response to the bone explants, the response of the osteocytes was investigated in these bone explants *ex vivo*.

Organ and cell culture systems have provided important insights regarding the response of bone cells to mechanical loading or to growth factors and hormones (Rawlinson et al. 1995, Cheng et al. 1996, Zaman et al. 1997). However, it has not been possible so far to maintain the tissue viable in culture due to their inability to mimic the in vivo bone microenvironment (Jones et al. 2001) resulting in tissue necrosis mainly in the centre of the organ culture (Jones et al. 2001, Davies et al. 2004). Furthermore, very little is known regarding the viability of osteocytes, considered to be the mechanosensors (Lanyon 1993, Zhang et al. 1997, Noble et al. 2003) and transducers in bone (Lean et al. 1996, Aarden et al. 1996, Duncan and Turner 1995, Klein Nulend et al. 1995, Yellowley et al. 2000) based on the ex vivo organ culture systems available to date. However, studies on bovine bone using the Zetos™ bioreactor have previously been shown to improve osteocyte viability by two fold in response to mechanical stimulation compared to the unloaded conditions following 28 days in culture (Mann et al. 2004). In this study the Zetos™ bioreactor was also employed in order to investigate possible beneficial effects of

mechanical loading on osteocytes in the presence or absence of, 17β-estradiol in human bone following 27 days in culture.

In order to determine the viability of the bone core samples immediately following their extraction from the patient, osteocytes were examined by means of their LDH activity in the T0 samples. In addition, osteocytes were categorised into two zones (**Figure 6.3**), depending on their distance from the upper surface of the cores, in order to determine possible effects of the cutting process on osteocyte viability. It was observed that more viable osteocytes (per mm^2) were present in Zone 2 compared to Zone 1 in the T0 cores possibly suggesting that the cutting process might have induced the loss of osteocytes observed in the latter. However, despite this loss of osteocytes in Zone 1, the density of osteocytes per mm^2 in both zones (125-150 osteocytes per mm^2 in Zone 1 and 200-250 osteocytes per mm^2 in Zone 2) was found to be within the range of viable osteocytes per mm^2 reported for human trabecular bone in the literature (Mullender et al. 1996, Tomkinson et al. 1998, Qiu et al. 2002).

Although the viability of osteocytes in the T0 was found to be within the **lowest** range of viable osteocytes per mm^2 reported (125-250 osteocytes/mm^2), the maintenance of bone explants under unloading conditions in the Zetos^{TM} bioreactor resulted in a dramatic decrease in the density of viable osteocytes (30 osteocytes/mm^2) after 27 days in culture. However, application of mechanical loading improved the viability of osteocytes in the human bone core explants compared to the unloaded samples by 1.5 fold. Studies in other ex vivo organ cultures of bovine trabecular bone demonstrated the inability of mechanical stimulation to improve osteocyte viability after 22 days in culture possibly due to insufficient nutrient perfusion to osteocytes in this ex vivo bone explant model (Takai et al. 2004). In contrast to these findings, mechanical loading applied in the Zetos^{TM} bioreactor appeared to maintain the viability of osteocytes to levels higher than those observed in the unloaded samples both at short-term (Mann et al. 2006) and long-term experiments as presented in this chapter.

In this study, the viability of cells was examined by means of their metabolic activity. More specifically, the metabolic activity of the LDH enzyme was monitored following the addition of tetrazolium salts to cells (§3.2.3), which are reduced in viable cells to a coloured product by the action of dehydrogenases (Tisserat and Manthey 1996, Verleysen et al. 2004). Based on this assay, only the living osteocytes were coloured because the colour observed is a product of metabolic activity in cells (Widholm 1972), whereas the non-coloured osteocytes were considered dead. These findings suggested that the measurement of viable cells per mm^2 in this study included only cells that were metabolically active. Although the estimation of LDH positive cells, as described above, would exclude the non-viable (necrotic) cells, it might be possible that apoptotic cells would be included in the viable pool measurements. This is based on evidence that suggests that apoptosis is an active, ATP-dependent process (Kerr 1972) and therefore cells at the early stages of apoptosis induction would still be metabolically active (Gramaglia et al 2004) and would possibly demonstrate LDH activity. Therefore, the response of osteocytes to the different treatments was further characterized by examining indices of early apoptosis based on an in situ DNA fragmentation technique.

Estimation of osteocyte apoptosis based on DNA fragmentation in the T0 samples indicated levels of apoptosis similar to levels reported in the literature for control human samples (Tomkinson et al. 1997). Maintenance of human bone in the Zetos^{TM} bioreactor in the absence of mechanical stimuli for 27 days resulted in an increase in osteocyte apoptosis by 627% in Zone 1 and by 436% in Zone 2 compared to the levels of apoptotic osteocytes in the T0 samples. This finding is in accordance with other studies showing that absence of mechanical loading resulted in an increase in osteocyte apoptosis (Noble et al. 2003, Bakker et al. 2004, Aguirre et al. 2006, Basso et al. 2006).

Furthermore, application of mechanical loading, which produced strains in the Zetos^{TM} bioreactor within the known osteogenic range (Mann et al. 2006), reduced osteocyte apoptosis compared to the unloading conditions in human bone. These findings are in accordance with previous studies that suggested that the application of physiological loading not only is beneficial for the maintenance of osteocyte viability in rat bone either

in vivo (Noble et al. 2003) or ex vivo (Lozupone et al. 1996) but are capable of regulating osteocyte viability and apoptosis in the human bone. In the absence of mechanical stimulation, the percentage of apoptotic osteocytes was estimated to be higher inside the bone cores. This finding could suggest that the perfusion of media alone without the presence of mechanical loading might not be enough to prevent osteocyte apoptosis in the middle of the cores and therefore mechanical stimulation is required.

A number of studies have suggested that estrogen might be affecting the response of bone to mechanical stimuli by lowering the threshold of sensitivity to loading (Frost 1988, Lanyon 1996). In addition, bone loss associated with estrogen deficiency might also be related to impaired responsiveness to mechanical stimulation caused by the absence of estrogen (Lanyon 1996). In this study, the effect of 17β-estradiol on the osteocytic population was investigated under disuse conditions or physiological loading stimuli in the human bone. Administration of 17β-estradiol did not decrease the levels of osteocyte apoptosis induced in response to the unloading conditions. Although estrogen is usually associated with beneficial effects on osteocyte viability (Tomkinson et al. 1997, 1998, Kousteni S et al., 2001) there are no previous reports in the literature to demonstrate the effects of estrogen on osteocyte apoptosis when osteocytes are subjected to mechanical stimulation. Findings reported in this thesis indicate that 17β-estradiol was unable to prevent osteocyte apoptosis induced by disuse situations in the human bone under the conditions investigated. In fact, it was observed that not only apoptosis was not prevented by 17β-estradiol in response to disuse conditions, but also that the anti-apoptotic effects exterted by mechanical stimulation were abolished in the presence of 17β estradiol in the inner zone. These findings indicated the possibility that 17β-estradiol could exert differential effects on the response of osteocytes to the disuse and loading conditions. However, the difference in this response according to what zone of the bone core was examined is very difficult to be explained and surely has to be investigated in the future in order to exclude human error or other reasons such as intra-sample variability.

Further studies are therefore required in order to determine the significance of these pro-apoptotic stimuli exerted by estrogen in the presence of loading on osteocytes. These

observations should be interpreted with caution since only one concentration of 17β-estradiol was employed in this study due to the limited number of cores obtained from the patient. For example, it might be possible that the concentration used in this study could have exerted toxic effects to the cells or it could have sensitised the cells to undergo apoptosis in response to the particular loading regime. A higher sample size would have allowed the use of different concentrations of estrogen as well as loading regimen. Therefore, further studies need to be conducted in order to determine the dose-dependency of the effects of 17β-estradiol on osteocytes in response to mechanical stimulation.

Histomorphometric analysis following administration of 17β-estradiol in the unloaded samples demonstrated a significant increase in the BFR compared to the unloaded samples that did not receive 17β-estradiol. As previously discussed in relation to the effects of mechanical stimulation, this finding possibly resulted from the increase in MAR and % pdL/BS pointing to a possible direct action of estrogen on the osteoblastic population/activity. The effects of estrogen and/or mechanical stimulation on the number and activity of osteoblasts could have been investigated by the use of the alkaline phosphatase staining that gives an indication of bone formation in response to mechanical stimulation.

Furthermore, studies have shown that bone formation induced by the administration of 17β-estradiol can occur independently of increased bone resorption (Lean et al. 1993). However, it is not known whether osteoclastic activity could have preceded bone formation by the osteoblasts since investigation of earlier time points was not included in this study. Further studies are required to address the presence of osteoclasts on these bone surfaces at this late time of investigation. This study did not cover the effects of estrogen and/or loading on osteoblasts or osteoclasts, which is the subject of future studies. Histological examination of the bone with phenotypic characterisation of the osteoblasts and osteoclasts would provide additional evidence that the Zetos system can maintain the viability of bone tissue during the experimental period. However, the use of frozen sections required in this study for the Nick Translation technique do not lend

themselves to the detailed histological analysis required for the study of bone surface-resident cells.

Furthermore, it is interesting to note that the same samples characterised by an increase in BFR, MAR and % pdL/BS in response to 17β-estradiol treatment were also characterised by high levels of apoptotic osteocytes. A number of studies are available to indicate that apoptotic osteocytes might be producing signals that direct the remodelling process (Verborgt et al. 2002, Noble et al. 2003) to specific areas in bone by increasing the osteoclastic activity. The perceived increase in osteocyte apoptosis in response to 17β-estradiol might have resulted from a reduced clearance of the apoptotic material due to a possible direct action of 17β-estradiol on the osteoclastic population. Studies have shown that 17β-estradiol exerts negative effects on osteoclast formation and survival (Girasole et al. 1992, Hughes et al. 1996, Kameda et al.1997), although such a possibility could not have been explored in this study due to the absence of osteoclasts at the later stages of investigation. In addition, the increase in osteocyte apoptosis due to the administration of 17β-estradiol in the presence of mechanical loading did not seem to affect osteocyte viability. This finding could be explained by the fact that the density of LDH-positive (viable) osteocytes is a cumulative effect over the 27-day culture period. On the contrary, the percentage of the apoptotic osteocytes presented in this study reflects a phenomenon that occurs at the time of the examination.

Other studies in bone have also reported a high incidence of apoptotic osteocytes during growth (Noble 1997), or in response to glucocorticoid (Weinstein et al. 1998) or GNRH treatment (Tomkinson et al.1997). These findings indicate that apoptotic osteocytes might not be rapidly removed from a site and might persist in their lacunae for prolonged periods of time. In fact, it has been shown in previous studies that dead osteocytes might remain in their lacunae for 16 weeks (Kenzora et al. 1978). This phenomenon of delayed removal of apoptotic osteocytes might be attributed either to the inaccessibility of osteoclastic cells to the mineralized matrix or to the low rate of turnover observed in human bone (Tomkinson et al. 1997). This might suggest that the time course of completion of the apoptotic process in osteocytes in the mineralised bone matrix is

the addition of 17β-estradiol to the system resulted in the enhancement of bone formation by the presence of 17β-estradiol compared to the loading treatment alone (Jagger et al. 1996). Therefore, although 17β-estradiol did not suppress the ability of mechanical stimulation to activate bone formation in this study, the fact that it did not further enhance BFR or MAR as observed in the disuse situation might be attributed to the fact that both 17β-estradiol and mechanical loading were administered at the same time. Further studies are required in order to address different osteogenic responses to a time delay between 17β-estradiol and loading administration. In addition, it might be possible that different biochemical pathways are employed by 17β-estradiol in response to disuse and loading stimuli which might also account for the significant differences between BFR and MAR under disuse and loading conditions in the presence of 17β-estradiol.

In summary, this study has provided evidence that osteocytes undergo apoptosis in response to disuse conditions which could be improved following the application of physiological loading in the Zetos^{TM} bioreactor. Application of mechanical loading or administration of 17β-estradiol to the unloaded samples induced an osteogenic response in this patient in terms of bone formation accompanied by increased mineral production.

The use of the Zetos^{TM} bioreactor in this thesis has enabled the study of the response of the osteocytic population to mechanical stimulation in the presence or absence of 17β-estradiol, since it is difficult to obtain human bone biopsies pre- and post-exercise due to ethical issues. However, this is a pilot study and a number of limitations were identified both in the design of the experiment as well as in the analysis of the results. The study was based on samples obtained from a single patient and not from n=3 as indicated by the statistical power of the experimental design. In addition, variability existed between the number of samples analysed for each treatment group (n=3 or n=2) which might have accounted for the high error bars (variance from the mean value) that were observed in some of the treatment groups. The low number of samples employed in this study was affected by the limited number of cores obtained from the patient. It would be important to determine the effect of more patients and a higher sample size on the results presented in this chapter. Furthermore, this study is limited by the administration of only one

are required in order to address different osteogenic responses to a time delay between 17β-estradiol and loading administration. In addition, it might be possible that different biochemical pathways are employed by 17β-estradiol in response to disuse and loading stimuli which might also account for the significant differences between BFR and MAR under disuse and loading conditions in the presence of 17β-estradiol.

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In the absence of mechanical stimulation, the percentage of apoptotic osteocytes was higher inside the bone cores. This finding could suggest that the perfusion of media

without mechanical loading might not be enough to prevent osteocyte apoptosis (and therefore mechanical stimulation is required).

Furthermore, this study is limited by the administration of only one calcein label on day 6 of the experiment. All histomorphometric analyses based on the estimation of double-labelled surfaces were performed using a pseudo label and therefore results should be interpreted with caution. However, the histomorphometric software employed in this study (BIOQUANT OSTEO, USA) allows for such an estimation to take place. In addition, the Zetos system does not come close to in vivo situations due to the lack of blood vessels. Diffusion might not be enough to ensure exchange of nutrients or waste metabolites between the cells away from the vessels. The bone marrow is not renewed and therefore it is difficult to investigate bone growth in this system following 27 days in culture.

Data in this chapter have suggested that, despite the low numbers of viable osteocytes present in the human bone explants following 27 days in culture, osteoblasts appeared to be responsive to the administration of 17β-estradiol or application of mechanical loading. Taking into account studies that suggest that osteocytes might be regulating the remodelling process (Noble et al. 2003, Power et al 2002) and that necrotic bone is poorly remodelled (Kenzora et al. 1978) these findings possibly point to a minimum number of osteocytes required in order to obtain an osteogenic response to loading.

CHAPTER 7

Conclusions and future work

Conclusions and future work

Withdrawal of estrogen either due to menopause or ovariectomy has previously been shown to induce bone loss and to be associated with osteocyte apoptosis *in vivo*. Data presented in this thesis, demonstrated a protective effect exerted by 17β-estradiol on the viability of murine osteocytes *in vitro* and rat osteocytes *in vivo*. However, administration of 17β-estradiol to ex vivo cultures of human bone in the Zetos bioreactor resulted not only did it not protect osteocyte viability but it resulted in the increase of osteocyte apoptosis as well. This finding could be explained by the fact that 17β-estradiol might employ different biochemical pathways in response to different stimuli such as for example estrogen loss or disuse.

However, no specific effects of SERMs have been associated so far with osteocyte viability. Data in this thesis have provided evidence that demonstrate for the first time that besides 17β-estradiol, the SERM LY 117018 also exerted protective effects on the osteocytic population in a rat model of ovariectomy. This finding has suggested that SERMs may play an important role in the maintenance of osteocyte viability, which might in the future contribute to the clinical development of estrogen replacement compounds with activities consistent with the maintenance both of bone mass and bone quality. Further studies are however required in order to determine a possible association between osteocyte apoptosis and bone loss using histomorphometric analysis, as well as the effects of SERMs on either or both of these processes.

This thesis has also provided novel evidence for the potential action of SERMs and estrogen as direct antioxidants, as part of their preventive mechanism against H_2O_2-induced osteocyte apoptosis. These findings are particularly important taking into account evidence that suggests that the accumulation of ROS, and specifically of H_2O_2 observed in ovariectomised mice (Lean et al. 2003), might be responsible for mediating the estrogen deficiency–induced bone loss (Lean et al. 2005). They also point to the need for further investigation into the antioxidant potential of compounds administered for the treatment of postmenopausal osteoporosis.

Although several studies have demonstrated the antioxidant capabilities of 17β-estradiol in various tissues *in vivo*, it remains to be answered whether SERMs also possess antioxidant properties *in vivo* and the possible association of these properties with the prevention of bone loss. This might be enabled for example by the measurement of oxidative stress markers in samples of urine/plasma along with markers of bone turnover. Further experiments are also required to determine any possible changes in the levels of antioxidant enzymes, as part of the protective effects of 17β-estradiol or SERMs, on osteocytes against oxidative stress.

In addition, it might be interesting to investigate any direct association between the increase in ROS production observed during estrogen loss and osteocyte apoptosis in vivo. Such an investigation would involve the use of ovariectomised mice, measurement of oxidative stress markers along with histochemical examination of apoptotic osteocytes. Futhermore, studies have shown that the inflammatory process associated with bone fractures is characterized by increased accumulation of ROS (introduction) and osteocyte apoptosis. Therefore, fracture repair studies on ovariectomised animals could be important in order to investigate any positive effects of the antioxidant properties of estrogen and SERMs on accelerating fracture healing in vivo. This approach could have a potential application in the aged postmenopausal population and might also open up new therapeutic possibilities for fracture repair.

Furthermore, taking into account data in both chapters 4 and 5, it might be possible to suggest that antioxidant properties might indeed be implicated in the prevention of osteoporosis. In addition, besides 17β-estradiol and SERMs which were demonstrated in this thesis to possess antioxidant properties, it remains to be determined whether pure antioxidant molecules also have a beneficial effect on bone loss when administered alone.

In addition, this study has provided important information on the effects of 17β-estradiol and mechanical stimulation on osteocyte viability in human bone ex vivo, and has demonstrated that the Zetos^{TM} bioreactor might be a valuable system for the prolonged maintenance of bone samples in culture. Further questions regarding the time course of

osteocyte apoptosis might be answered by the use of the Zetos™ system which allows human trabecular bone biopsies to be examined over a time course. Future work should also explore whether SERMs exert any beneficial effects on human osteocytes ex vivo, similar to those observed in rat osteocytes in vivo and murine osteocytes in vitro (chapters 3, 4 and 5). Furthermore, the application of molecular biology techniques on samples maintained in the Zetos™ system might provide useful insight into the mechanisms and pathways employed by mechanical stimulation and/or estrogen or SERMs in the maintenance of osteocyte viability in human bone.

Previous studies have suggested that disuse conditions have been associated with an increase in oxidative stress in rat bone (Smith et al., 2005). Using the Zetos™ system it might be interesting to investigate the impact of mechanical stimulation and/or anti-osteoporotic drugs such as estrogen or SERMs on oxidative stress in human bone in association with osteocyte viability and bone histomorphometric indices. Possible changes in oxidative stress levels might be detected by the measurement of oxidative stress markers collected frequently from the medium of the samples maintained in the Zetos™ along with immunohistochemical examination of antioxidant enzymes. This might prove to be valuable in order to explore the potential novel action of estrogen and SERMs as antioxidants against postmenopausal bone loss.

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Publications

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The antioxidant effect of estrogen and Selective Estrogen Receptor Modulators in the inhibition of osteocyte apoptosis *in vitro*

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Abstract

Withdrawal of estrogen represents the primary factor determining post menopausal bone loss and has been associated with negative indicators of bone quality including the apoptotic death of osteocytes *in vivo*. While hormone replacement therapy in the form of Estrogen or Selective Estrogen Receptor Modulators (SERMs) demonstrates clear estrogen receptor (ER)-mediated benefits to bone mass, less is known regarding the mechanism of action of these compounds in the maintenance of bone cell populations. We have investigated the potential antioxidant effects of estrogen, estrogen derivatives and the SERMs Raloxifene and LY117018 in the prevention of oxidative stress induced apoptosis in the osteocyte like cell line MLO-Y4.

Treatment of MLO-Y4 with 0.3 mM H_2O_2 induced apoptosis that was significantly inhibited ($p \leq 0.002$) when the cells were pre-treated for 1 h with either 17β-estradiol, Raloxifene or LY117018 (10 nM). The stereoisomer 17α-estradiol also prevented H_2O_2 induced apoptosis in MLO-Y4. Importantly, pre-treatment of ER-negative HEK293 cells with either 1 μM, 100 nM or 10 nM 17β-estradiol, Raloxifene or LY117018 significantly inhibited H_2O_2 induced apoptosis in these cells ($p \leq 4.2 \times 10^{-5}$) indicating an estrogen receptor-independent effect of these compounds. Comparisons of 17β-estradiol and similar molecules containing the putative free radical scavenger C3-OH moiety on the steroid A-ring (17α-estradiol, 17α-ethinylestradiol; 10 nM) with structurally related molecules lacking the C3-OH grouping (Mestranol and Quinestrol; 10 nM) demonstrated that only compounds containing the C3-OH moiety showed anti-apoptotic behavior in these studies ($p \leq 0.0033$). Similarly the identification of the presence of reactive oxygen species (ROS) in cells as evidenced by the free radical indicator 2′7′-dichlorodihydrofluorescein diacetate demonstrated that 17β-estradiol, SERMs and related molecules with C3-OH moiety were capable of blocking ROS generated in cells by H_2O_2 ($p \leq 0.002$) while Mestranol and Quinestrol showed no such blockade. It is possible that the loss of osteocytes during estrogen insufficiency may occur through a failure to suppress the activity of naturally occurring or disease associated oxidant molecules. These data suggest that the osteocyte protective effects of estrogen and SERMs may operate through a common receptor-independent mechanism which may be related to the antioxidant activity of these molecules.

Keywords: Osteocyte; Apoptosis; Estrogen; SERMs; Antioxidant

Introduction

Clinically, osteoporosis is defined as a Bone Mineral Density (BMD) measurement of less than 2.5 standard deviations below the young adult mean (*T*-score); however, BMD alone cannot account for all of the variation in bone strength [1]. For this reason, measures of bone quality for example, bone architecture [2,3] and micro-damage accumulation [4] are now considered possible determinants of bone strength. Less well established is the obvious impact on bone quality that reduced skeletal cellularity or osteocyte viability might have. Osteocytes are the terminally differentiated cells of the osteoblast lineage which become entrapped within newly formed osteoid tissue during bone formation. Osteocytes are characterized by having dendrite-like cell processes within canaliculi that form a communication network between

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both neighboring osteocytes and effector cells at the bone surface [5]. The presence of viable osteocytes within bone has been shown to be associated with bones' ability to remodel efficiently [6–9], to maintain normal levels of mineralization and to repair accumulated microdamage [10,11]. For these reasons, it is likely that osteocyte viability represents an important and to date poorly understood determinant of bone quality. The number of viable osteocytes in the human femur decreases from 90% at age 1–10 years to 20% at age 90 years [12]. The reasons for the age related loss of osteocytes are unclear but it is known that osteocyte apoptosis is engendered by a wide variety of conditions including glucocorticoid excess [13], microdamage [14], estrogen loss [15] and oxidant attack [16].

The "free radical theory" of ageing is based on evidence suggesting that Reactive Oxygen Species (ROS) production and the response to oxidative stress contribute to a number of age related phenomena [17]. For example oxidative stress has been linked to the incidence of Alzheimer's Disease (AD) and Parkinson's disease [18]. Importantly, both *in vitro* [19] and *in vivo* [20] studies have reported neuroprotective effects of estrogen under conditions associated with oxidative stress induced neuronal cell death. These effects have been shown to be receptor-independent and attributed to the antioxidant properties of estrogen related to the presence of a hydroxyl group at the C3 position on the steroid A ring of the hormone [20]. In a similar way to neuronal cell system, the osteocyte population in bone is also responsive to estrogen. Estrogen deficiency has been shown to increase osteocyte death in human and rat bone [15] and, in ovariectomized rats, the ovariectomy induced stimulation of osteocyte apoptosis can be reversed upon estrogen replacement [21]. To date the molecular mechanism by which estrogen acts to maintain the osteocyte population has not been fully elucidated. While the classical mode of action of estrogen is through gene activation after binding to specific nuclear receptors, it is now clear that estrogen can elicit a variety of non-genomic biological effects. Estrogen binding to specific receptors results in the activation of G-proteins [22], MAP kinase signaling pathways [23] and PI3K signaling pathways [24]. The Selective Estrogen Receptor Modulators (SERMs) are non-steroidal molecules with tissue specific receptor-mediated effects on various target tissues. They retain the positive effects of estrogen on bone without its associated adverse effects on breast and uterine tissue [25]. While the Raloxifene analogue LY117018 (Lilly) has been shown to protect osteocytes from ovariectomy induced apoptosis *in vivo* in a rat OVX model [26], the molecular mechanism of the protection has yet to be determined. While structurally different to estrogen, Raloxifene and the Raloxifene analogue LY117018 do retain a cyclohexane C3 hydroxyl grouping which could potentially facilitate antioxidant activity. The purpose of this study was to investigate the possibility that estrogen and SERMs act as antioxidants during oxidative stress engendered induction of apoptotic cell death in osteocyte like cells, with a view to understanding the potential use of these compounds in the improvement of both the quantity and quality of bone.

Materials and methods

Materials

All chemicals were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. Estrogen-like compounds used in this study included 17β-estradiol (Calbiochem), the stereoisomer17α-estradiol, the estrogen receptor antagonist ICI 182, 780 (Tocris Cookson, Ltd.), estrogen derivatives ethinylestradiol 3-methyl ether (Mestranol), ethinylestradiol 3-cyclopentyl ether (Quinestrol), 17α-ethinylestradiol and SERMs Raloxifene and Raloxifene analogue LY117018 (a gift from Eli Lilly) (Fig. 1). The antioxidant Vitamin E was used to investigate the potential cell protective activity of a potent antioxidant compound in this model system.

Cell culture

The MLO-Y4 osteocyte like cell line [27] (a gift from Prof. Lynda Bonewald, San Antonio) was cultured at a density of 2.4×10^4 cells per ml on collagen (Type I Rat Tail) coated 24 well plates in alpha modified essential medium (α-MEM, Gibco UK) supplemented with 5% FBS, 5% NCS, penicillin (50 I.U./ml), streptomycin (50 μg/ml) and L-Glutamine (2 mM). The HEK293 human embryonic kidney epithelial cell line (obtained from Prof. Phillipa Saunders, MRC Reproduction Unit, University of Edinburgh) was cultured at a density of 2.4×10^4 on 24 well plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, penicillin (50 I.U./ml), streptomycin (50 μg/ml) and L-Glutamine (2 mM). Pre-treatment of cells with estrogen, estrogenic derivatives, SERMS and Vitamin E was carried out for 1 h prior to induction of oxidative stress. The pre-treatment agents remained in culture during H_2O_2 induced oxidative stress. In experiments using either the estrogen receptor antagonist ICI 182780 or ERK1/2 inhibitor UO126 (Promega), the cells were pre-incubated for 1 h with ICI 182780 (10 nM) or 30 min with UO126 (20 μM) which remained in culture prior to the further addition of estradiol, estradiol derivatives or SERMs and also throughout the induction of oxidative stress. At the end of the experimental period, the cells were washed with PBS and fixed in 4% paraformaldehyde solution.

Oxidative stress induced cell death

Oxidative stress was induced in cells by incubation with H_2O_2 (30% v/v) within a concentration range of 0.08 mM–0.6 mM. The induction of apoptotic cell death was monitored microscopically using morphological criteria.

Identification of apoptosis

Cells were stained using DAPI (1 μg/ml) nuclear stain and apoptotic cells were characterized by cell shrinkage, plasma membrane blebbing (transmitted light) and nuclear condensation (fluorescent 358 nm excitation, emission 461 nm). Necrotic cells were identified by rapid (within 2 h) cell swelling and/or bursting and loss of membrane integrity. Cell numbers were determined following H_2O_2 treatment in order to monitor the rapid cell death characteristic of necrosis. Digital images of cells were captured at 20× magnification using an inverted microscope fitted with DXM 1200 camera (Nikon UK Limited). For analysis, $n=3$ wells per treatment and six fields per well were analyzed using Scion Image Software (Scion Corporation, USA). Within each field, the total cell number and the number of apoptotic cells were assessed and the results were expressed as the percentage apoptosis ± standard deviation (SD).

Inhibition of caspase 3/7 activity

In order to further demonstrate the apoptotic nature of the oxidant engendered response, a specific caspase 3/7 inhibitor (GlaxoSmithKline, USA) which is based on Isatin Sulfonamides [28] was used to inhibit terminal effector caspases 3 and 7. Cells were pre-incubated with caspase 3/7 specific inhibitor at a concentration of 1 M for 1 h prior to the addition of 0.3 mM H_2O_2. At the end of the experimental period, the cells

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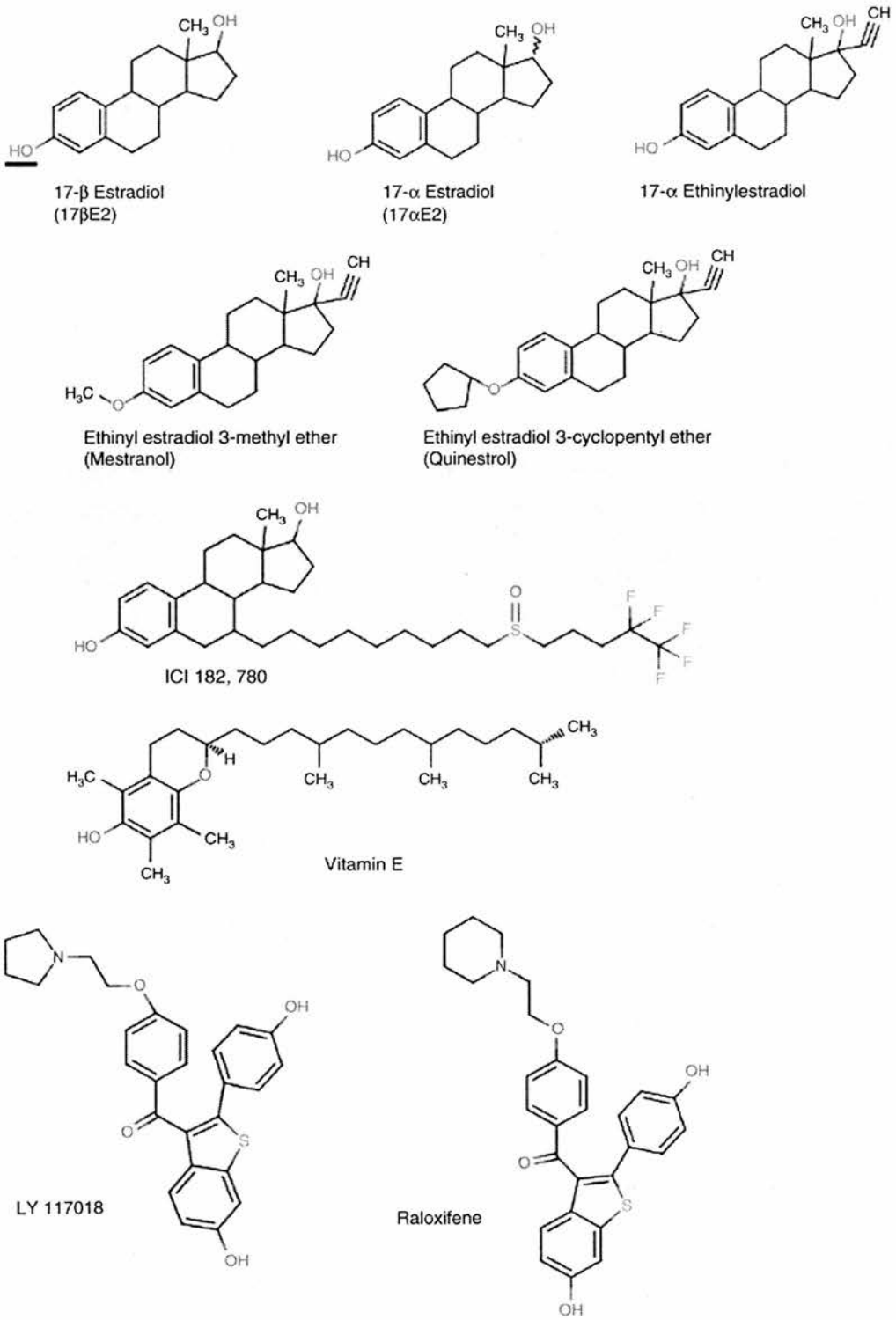

Fig. 1. The presence of an aromatic phenol group containing a free hydroxyl –OH group facilitates the reaction with oxyradicals by H-atom transfer due to the relatively weak O–H bond of the hydrogen group. This C3-OH grouping is common to the basic steroid hormone A-ring of vitamin E, 17β-estradiol, stereoisomer 17α-estradiol and antiestrogen ICI 182 780. Mestranol, Quinestrol and 17α-ethinylestradiol are steroidal derivatives of estradiol; however, Mestranol has a CH_3O-methyl substitution and Quinestrol a cyclopentyl substitution on the C3 A-ring position, while the SERMs Raloxifene and LY117018 lack the steroidal structure of estrogen they retain the phenolic C3-OH group.

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were washed with PBS and fixed in 4% paraformaldehyde solution and the percentage apoptosis was assessed microscopically as described previously.

Western blot analysis

Cell lysates were prepared from MLO-Y4 cells following treatment for 2 min with 17β-estradiol, Raloxifene and LY117018 (10 nM) in the presence and absence of UO126 (20 μM). The lysis buffer contained 20 mM Tris–HCl, pH 7.5, 0.1% (v/v) Igepal, 6 mM sodium deoxycholate, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 0.1 mM Na_3VO_4, 20 mM NaF and a protease inhibitor cocktail tablet (Roche, UK). Protein concentrations were estimated using a commercially available kit (Bio-Rad, UK) and lysates (35 μg/lane) were resolved on 12% SDS-PAGE gels and transferred onto PVDF membranes and blocked with TBST solution (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) supplemented with 3% BSA prior to hybridization with a rabbit polyclonal antibody against phospho-p44/42MAPK (New England Biolabs, UK). Proteins were detected using Enhanced Chemiluminescence (ECL Amersham) according to the manufacturer's instructions. The blots were stripped (100 mM â-mercaptoethanol, 69 mM SDS and 62.5 mM Tris–HCl, pH 6.7) and rehybridized with an antibody that recognizes total p44/42 MAPK to verify equal loading of samples. Cell lysates were obtained from HEK293 along with MCF-7 epithelial breast cancer cell line and ER-α transfected HEK293 cells as positive controls (all obtained from Prof. Phillipa Saunders, MRC Reproduction Unit, University of Edinburgh). Western blots were prepared as described above and probed using specific mouse monoclonal antibody against ERα (Novocastra).

Reverse transcription PCR

Total RNA was extracted from Human Ishikawa Endometrial cells (ISH) (obtained from Prof. Phillipa Saunders, MRC Reproduction Unit, University of Edinburgh) and HEK293 using RNAeasy kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized with Oligo dT primers using Bioscript reverse transcriptase (Bioline) and PCR performed using the specific primers sequences listed below for ERα, ERβ and GAPDH at a final concentration of 0.5 μM in RedTaq reaction mix (Bioline) at an annealing temperature of 55°C.

ER-α	S 5′-AGCACCCTGAAGTCTCTGGA-3′
	AS 5′-GATGTGGGAGAGGATGAGGA-3′
ER-β	S 5′-TCAGGCATGCGAGTAACAAG-3′
	AS 5′-GCTTTTACTGTCCTCTGCCG-3′
GAPDH	S 5′-GTCAAGGCTGAGAACGGGAA-3′
	AS 5′-GCTTCACCACCTTCTTGATG-3′

Detection of intracellular reactive oxygen species

Detection of cells containing significant levels of reactive oxygen species (ROS) was determined after incubation of cells with 2′,7′-dichlorodihydro-fluorescein-diacetate (H_2DCF-DA). H_2DCF-DA is a cell permeable non-fluorescent probe that is de-esterified intracellularly and turns to a highly fluorescent 2′,7′-dichlorodihydrofluorescein upon oxidation by intracellular H_2O_2 and ROS. H_2DCF-DA (10 μM) was added to the culture medium 30 min prior to incubation with either estrogen, estrogenic derivatives, SERMs or Vitamin E or vehicle. The cells were then incubated with these agents for 1 h prior to treatment with H_2O_2 (0.3 mM for 2 h). The H_2DCF-DA and pre-treatment agents remained in culture during H_2O_2 treatment after which the media were removed and the cells washed twice with PBS before re-addition of α-MEM supplemented with 5% FBS, 5% NCS, penicillin (50 I.U./ml), streptomycin (50 μg/ml) and L-Glutamine (2 mM). Live cells were monitored using an inverted microscope fitted with an environmental chamber to maintain the temperature at 37°C and 5% humidified CO_2. ROS positive cells were detected at 490 nm FITC excitation and images captured at 20× magnification using DXM1200 color camera. The cells were monitored over a period of 2.5 h based on preliminary experiments determining the time course of ROS induction (data not shown). Digital images were analyzed using Scion Image Software. For analysis, $n=3$ wells per treatment and six fields per well were counted.

Statistical analysis

Statistical differences between treatment groups were determined using one-way ANOVA followed by Tukey–Kramer test post hoc and results were considered significant when $p<0.05$ denoted by * when the experimental treatment was compared to H_2O_2 treatment, and $p<0.05$ denoted by † when the experimental treatment was compared to vehicle control. The results presented here are representative of $n\geq 3$ experiments.

Results

Oxidative stress induced apoptosis

H_2O_2 induced apoptosis in osteocytes in a dose-dependent manner over a 2 h incubation period. An increased proportion of apoptotic cells compared to control were observed at H_2O_2 concentrations as low as 0.08 mM (% apoptosis 13.6±4.4 versus control 2.7±2.6; $p=1.18\times10^{-5}$) and was maximally induced at H_2O_2 concentrations of 0.3 mM (% apoptosis 31.7±7.4; $p=6.5\times10^{-9}$) (Fig. 2A). Based on cell morphology, both apoptosis and necrosis were obtained after treatment with H_2O_2 concentrations of 0.4 mM and above. The evidence for necrosis was confirmed by cell swelling/bursting and the rapid (within 2 h) loss of cell number at the higher concentrations of H_2O_2 (cell number 0.4 mM H_2O_2 14±7.5 versus control 29±6.7; $p=0.00043$) (Fig. 2B). In contrast, H_2O_2 concentrations that induced the morphological characteristics of apoptosis did not alter cell numbers within 2 h (Fig. 2B). Apoptosis inducing concentrations of H_2O_2 did result in a reduction in cell numbers 24 h after treatment as would be expected following the initiation of the apoptotic cascade (cell number 27.03±1.2 in control versus 9.2±1.07 in 0.3 mM H_2O_2 treated cultures; $p=0.0001$). Pre-incubation of the cells for 1 h with the specific caspase 3/7 inhibitor at 1 μM, which we have shown previously to block osteocyte apoptosis [13], prior to H_2O_2 treatment at 0.3 mM significantly reduced the percentage of apoptotic cells from 16.5±2.02 to near control levels of 4.4±1.04 ($p=0.0017$) confirming an apoptotic route to cell death under these conditions (Fig. 2C). Based on these results, a concentration of H_2O_2 of 0.3 mM for 2 h was used to induce apoptosis in the subsequent experiments.

Reduction in the percentage of apoptotic osteocytes on pre-treatment of cells with 17β-estradiol, SERMS and vitamin E

The percentage of H_2O_2 induced apoptotic osteocytes (17.7±2) was significantly reduced after pre-treatment of cells for 1 h with 17β-estradiol at concentrations of 1 μM (5.8±2; $p=0.01$), 100 nM (4.3±1.7; $p=0.007$) and 10 nM (2.5±0.6; $p=0.002$) (Fig. 3A). When added alone, the high 1 μM concentration of 17β-estradiol significantly induced apoptosis in cells compared to control cultures (6.4±2.8 versus 1.5±0.5 respectively; $p=0.016$) (Fig. 3A). Since the lower, near physiological concentration of 17β-estradiol (10 nM) reduced H_2O_2 induced apoptosis without demonstrating a pro-apoptotic effect on osteocytes, this concentration was used in sub-sequent treatments.

At a concentration of 10 nM comparable to the optimal anti-apoptotic estrogen concentration, both Raloxifene and

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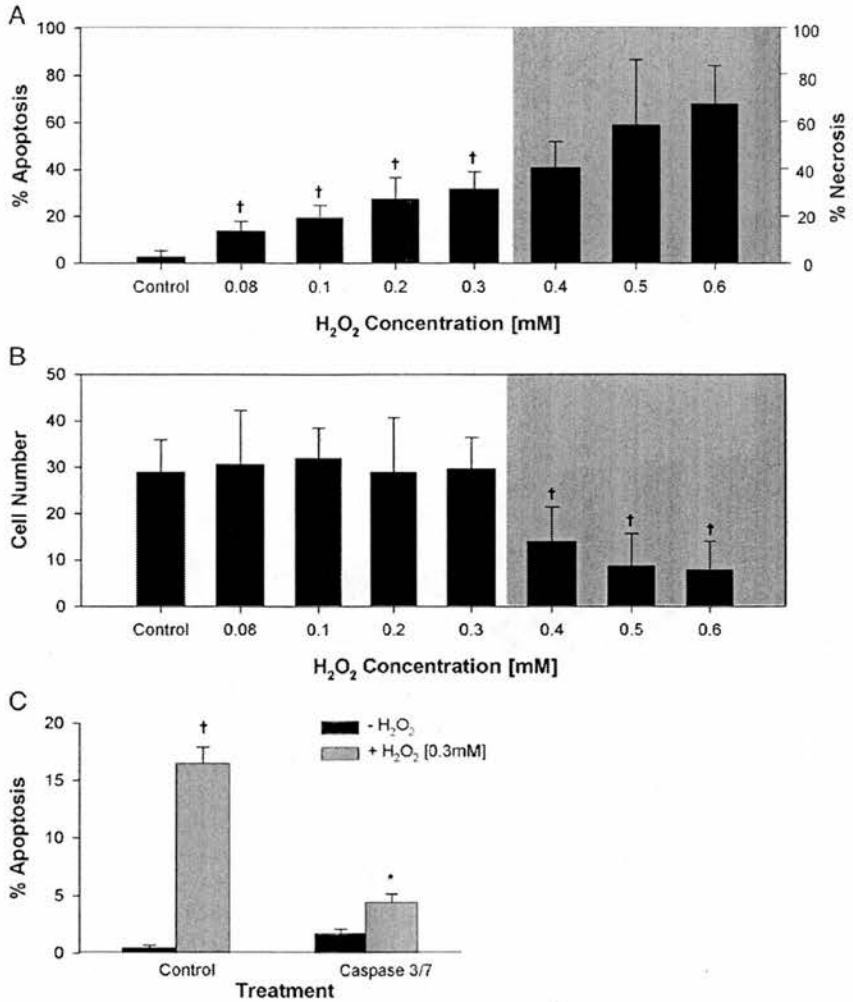

Fig. 2. A 2 h incubation of MLO-Y4 with varying concentrations of H_2O_2 between 0.08 and 0.6 mM was used to induce oxidative stress cell death. (A) The percentage of apoptotic cell death increased significantly compared to control between 0.08 mM and 0.3 mM H_2O_2. At H_2O_2 concentrations of 0.4 mM and above, the cells showed morphology characteristic of necrosis (shaded area). (B) At H_2O_2 concentrations up to 0.3 mM, there was no change in cell numbers; however, at 0.4 mM and above, cell numbers were significantly reduced compared to control indicative of necrotic rather than apoptotic form of cell death. (C) Inhibition of Caspase 3/7 caused a reduction in the percentage apoptosis following H_2O_2 treatment confirming an apoptotic route to cell death. Bars indicate means ± SD of triplicate wells (six fields per well counted); $^{\dagger}p<0.05$ versus vehicle control and $^{*}p<0.05$ versus H_2O_2 treatment by ANOVA.

LY117018 significantly reduced H_2O_2 induced apoptosis ($p \leq 0.0003$) (Fig. 3B). Treatment of cells with 1 μM and 100 nM concentrations of Raloxifene and LY117018 showed no pro-apoptotic effect while significantly reducing ($p \leq 0.0003$) H_2O_2 induced apoptosis (Fig. 3B). The percentage of H_2O_2 induced apoptotic osteocytes (20±2) was significantly reduced after pre-treatment of cells for 1 h with vitamin E at concentrations of 100 μM (3.85±0.09; $p=0.00023$), 1 μM (3.44±1.74; $p=0.0005$) and 10 nM (1.53±0.06; $p=0.0009$) (Fig. 3C).

ERK 1/2 inhibition

In order to determine whether the saving effects of estrogen and SERMs were mediated via receptor activation of MAP kinase signaling pathways, we used the MEK inhibitor UO126. U0126 is a chemically synthesized organic compound that inhibits activation of MAPK (ERK 1/2) by inhibiting the kinase activity of MAP kinase kinase (MAPKK or MEK 1/2) [29]. Dose dependency experiments have previously shown that optimal inhibition of ERK in the absence of unwanted cell viability side effects in MLO-Y4 cells is achieved using a UO126 concentration of 20 μM [13]. UO126 was shown to inhibit ERK1/2 activation following 2 min pre-treatment of MLO-Y4 using either 10 nM 17β-estradiol, Raloxifene or LY117018 (Fig. 4A). ERK activation via estraõdiol at this time point has been shown previously by Kousteni et al. [30]. Pre-treatment of MLO-Y4 with UO126 (20 μM) prior to the addition of 17β-estradiol did not inhibit the anti-apoptotic effects of 17β-estradiol (% apoptosis UO126+H_2O_2 15.7±1.36 versus % apoptosis UO126+17β-estradiol+H_2O_2 6.15±1.69; $p=0.0088$) or Raloxifene (% apoptosis UO126+Raloxifene+H_2O_2 3.42±0.66; $p=0.00120$) and LY117018 (% apoptosis UO126+LY117018+H_2O_2 8.4±2.57; $p=0.037$) (Fig. 4B).

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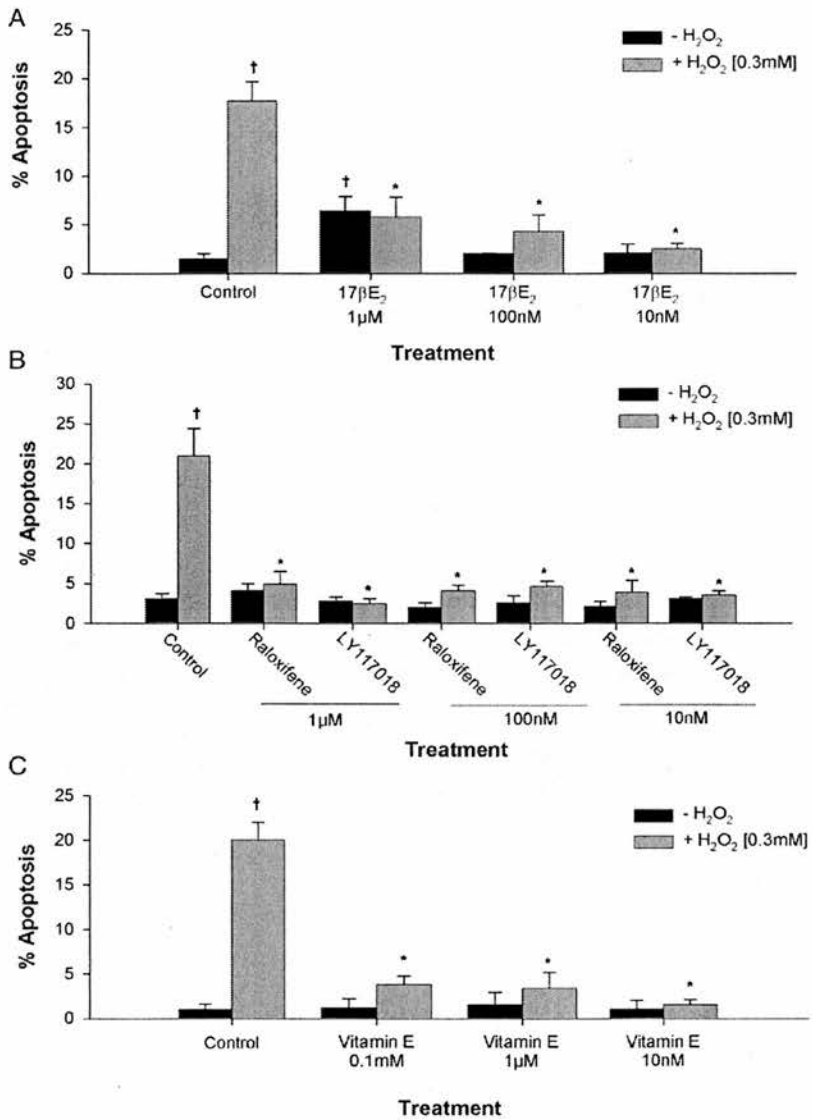

Fig. 3. (A) Pre-treatment of MLO-Y4 with 17β-estradiol (17-βE$_2$) at concentrations of 1 μM, 100 nM and 10 nM all significantly reduced the percentage of apoptosis induced by 0.3 mM H_2O_2. The pharmacological concentration of 17-βE$_2$ (1 μM) was found to induce apoptosis of MLO-Y4 when compared to control. (B) Pre-incubation of cells with SERMs Raloxifene and Raloxifene analogue LY117018 at concentrations of 1 μM, 100 nM and 10 nM also significantly reduced the percentage of H_2O_2 induced apoptosis. (C) H_2O_2 induced apoptosis was significantly reduced on pre-treatment of osteocytes with potent antioxidant vitamin E at concentrations of 0.1 mM, 1 μM and 10 nM. Bars indicate means±SD of triplicate wells (six fields per well counted); $^{\dagger}p<0.05$ versus vehicle control and $^{*}p<0.05$ versus H_2O_2 treatment by ANOVA.

Non-receptor-mediated anti-apoptotic effects

In order to determine whether any anti-apoptotic effects of these test compounds were mediated via the nuclear estrogen receptor, we initially used the receptor antagonist ICI 182,780 which is an estradiol derivative containing an alkylamide extension in the 7α position. Previous studies have shown that ICI 182,780 acts to disrupt the receptor shuttling process by inhibiting nuclear uptake of the receptor [31]. Pre-treatment of cells with the receptor antagonist ICI 182,780 (10 nM) prior to

Fig. 4. (A) UO126 at a concentration of 20 μM prevented activation of ERK1/2 induced after treatment of MLO-Y4 with either 10 nM 17β-estradiol or SERMs. (B) ERK 1/2 inhibition did not block the saving effects of 17-β Estradiol or SERMs on treatment of MLO-Y4 with H_2O_2. (C) Pre-treatment of MLO-Y4 with estrogen receptor antagonist ICI 182 780 did not block the saving effect of 17β-estradiol; pre-treatment of cells with the stereoisomer 17α-estradiol also reduced oxidative stress induced apoptosis. (D) ERα was not detected following Western blot analysis of HEK293 cells. MCF-7 and ERα transfected HEK293 are shown for positive controls. (E) Neither ERα nor ERβ was detected in HEK293 following RT-PCR. Ish cells are shown for positive control. (F) Pre-treatment of ER-negative HEK293 cells with either 17β-estradiol, Raloxifene or LY117018 at concentrations of 1 μM, 100 nM and 10 nM all significantly reduced H_2O_2 induced apoptosis. Bars indicate means±SD of triplicate wells (six fields per well counted); $^{\dagger}p<0.05$ versus vehicle control and $^{*}p<0.05$ versus UO126 treatment 4A and $^{*}p<0.05$ H_2O_2 treatment 4B and C by ANOVA.

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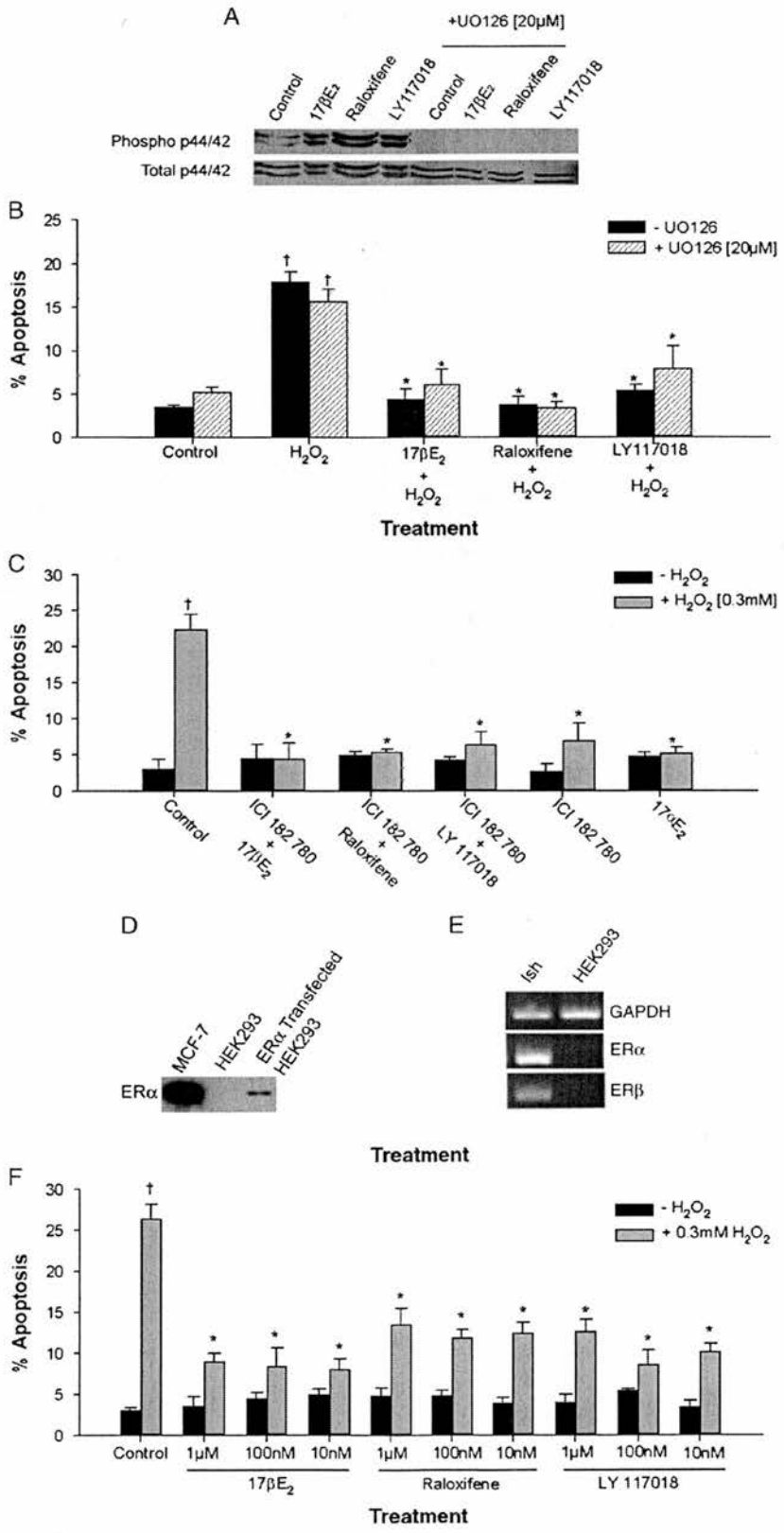

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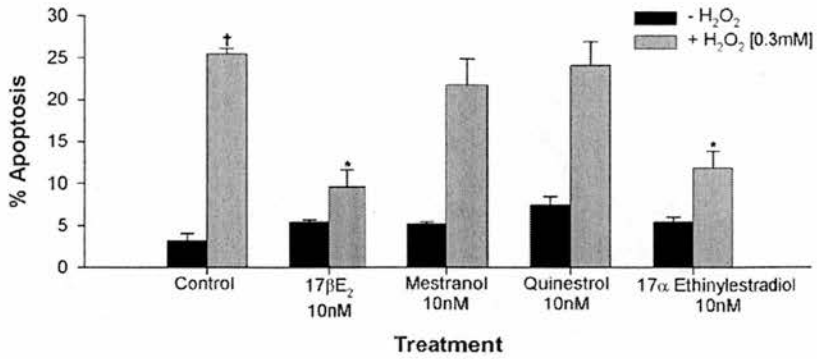

Fig. 5. Pre-treatment of MLO-Y4 with either Mestranol or Quinestrol which lack the C3-OH did not prevent H_2O_2 induced apoptosis while 17α-ethinylestradiol was capable of saving cells from H_2O_2 induced apoptosis. Bars indicate means±SD of triplicate wells (six fields per well counted); $^{\dagger}p<0.05$ versus vehicle control and $^{*}p<0.05$ versus H_2O_2 treatment by ANOVA.

the addition of 17β-estradiol did not inhibit the anti-apoptotic effects of 17β-estradiol (% apoptosis 4.5±2.2 versus H_2O_2 treatment 22.4±1.8; $p=0.0005$) (Fig. 4C). In a similar way, the anti-apoptotic effect of Raloxifene and LY117078 was not blocked by pre-incubation with ICI 182 780 (Fig. 4C). However, H_2O_2 induced apoptosis was also significantly reduced on pre-treatment of cells with ICI 182 780 (% apoptosis 7±2.4; $p=0.001$) in the absence of 17β-estradiol indicating that the ICI 182 780 compound has an anti-apoptotic activity in this model system (Fig. 4C). Pre-incubation of cells with the stereoisomer 17α-estradiol (10 nM) which does not activate ER-mediated transcription also resulted in a significant

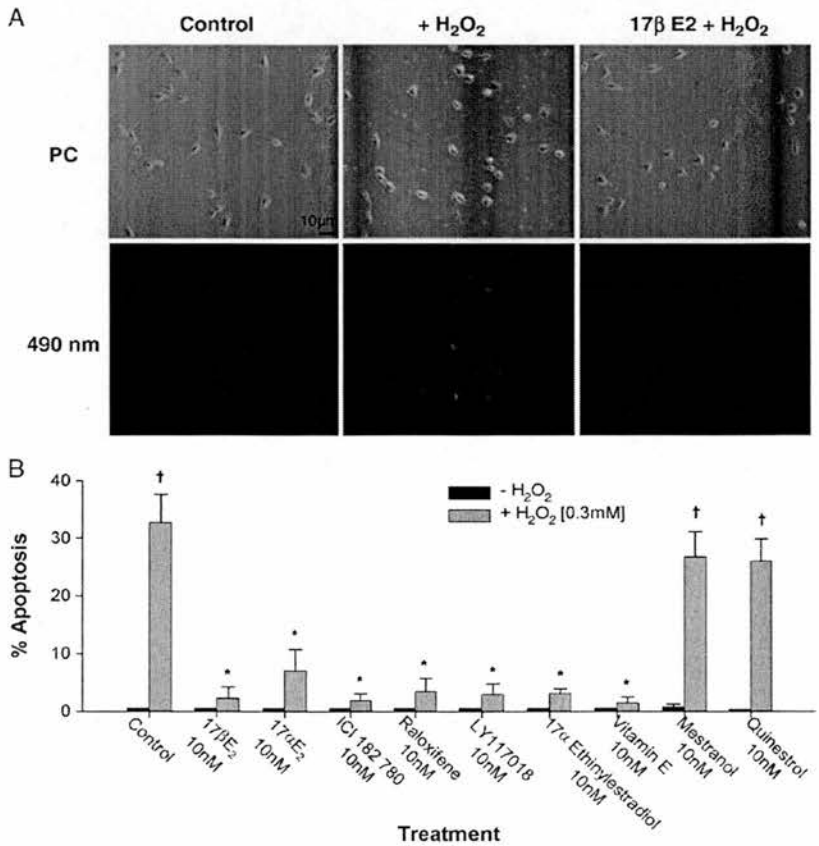

Fig. 6. The images shown are representative of phase contrast (top row) and H_2DCF-DA activation detected at 490 nm (bottom row). (A — left panel) Control MLO-Y4 (no treatment), (A — middle panel) MLO-Y4 following 2 h 0.3 mM H_2O_2 treatment and (A — right panel) MLO-Y4 following 1 h 17β-estradiol pre-incubation prior to the addition of 0.3 mM H_2O_2. Treatment of MLO-Y4 with H_2O_2 induced the production of ROS as detected by H_2DCF-DA (bottom row middle panel). (B) ROS activity was reduced on pre-treatment with either 10 nM 17β-estradiol, 17α-estradiol, SERMs, ICI 182,780, 17α-ethinylestradiol and vitamin E which all display C3-OH moiety. Mestranol and Quiestrol had no effect on the reduction of ROS positive cells on H_2O_2 treatment. Bars indicate means±SD of triplicate wells (six fields per well counted); $^{\dagger}p<0.05$ versus vehicle control and $^{*}p<0.05$ versus H_2O_2 treatment by ANOVA.

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reduction in the percentage osteocyte apoptosis when compared to H_2O_2 treatment (% apoptosis 22.4±2.1 H_2O_2 treatment versus 5.2±0.8 17α-E_2+H_2O_2 treatment; p= 0.0017) (Fig. 4C).

In order to further elucidate the potential contribution of estrogen receptor-mediated events in this response, we used the HEK293 human embryonic kidney epithelial cell line which is ER-α and ER-β negative (Figs. 4D and E) [32,33]. Administration of H_2O_2 induced apoptosis in HEK293 cells in a dose-dependent manner between 0.08 mM and 0.3 mM concentrations of H_2O_2 over a 2 h incubation period and apoptosis at levels comparable to that induced in MLO-Y4 osteocyte cell line was seen in HEK293 cells using 0.3 mM H_2O_2 incubation for 2 h (% apoptosis 0.3 mm H_2O_2 21.5±1.52 versus control 4.5±0.2; p=0.0012). The percentage of H_2O_2 induced apoptotic HEK293 cells (20.3±1.19) was significantly reduced after pre-treatment with 17β-estradiol for 1 h at doses of 1 μM (7.3±1.41; $p=5.3\times10^{-6}$), 100 nM (6.5±1.88; $p=8.1\times10^{-6}$) and 10 nM (3.09±0.41; $p=4.3\times10^{-8}$) (Fig. 4D). Pre-incubation of HEK293 cells with Raloxifene and LY117018 at concentrations of 1 μM, 100 nM and 10 nM also significantly reduced ($p\leq4.2\times10^{-5}$) the percentage of H_2O_2 induced apoptosis (Fig. 4F).

Antioxidant structure and function relationship

Pre-incubation of cells for 1 h with either the estrogen derivative Mestranol (10 nM) which has a CH_3O-methyl group or Quinestrol (10 nM) which has a cyclopentyl group at the C3 position of the steroid A-ring (not a hydroxyl group) failed to inhibit H_2O_2 apoptosis (% apoptosis H_2O_2 treatment 25.3±0.67 versus Mestranol+H_2O_2 21.7±3.1; p=0.32; Quinestrol+H_2O_2 24.1±2.7; p=0.69). While pre-incubation of cells with 17α ethinylestradiol, which is structurally similar to Mestranol and Quinestrol but with the -methyl and -pentyl groups respectively substituted by C3-OH, significantly reduced H_2O_2 induced apoptosis (% apoptosis 11.8±2; p=0.0033) (Fig. 5). The ability of H_2O_2 to generate reactive oxygen species in MLO-Y4 was investigated using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). The proportion of ROS positive cells was increased within 2 h of treatment with H_2O_2 (Fig. 6A). Pre-treatment with either 17β-estradiol, 17α-estradiol, ICI 182 780 and 17α-ethinylestradiol or SERMs at a concentration of 10 nM all significantly reduced ($p\leq0.003$) the proportion of ROS positive cells following treatment with H_2O_2 to levels similar to those seen in control cultures, as did the potent antioxidant vitamin E (10 nM) (Fig. 6B). In contrast to these data pre-incubation with Mestranol or Quinestrol at 10 nM concentration failed to reduce the number of ROS positive cells (Fig. 6B).

Discussion

Our data demonstrate that the treatment of osteocyte like cells with H_2O_2 caused oxidative stress engendered cell death with the morphological characteristics of apoptosis at concentrations between 0.08 mM and 0.3 mM. These data are in agreement with the work of Kikuyama et al. [16] in which similar concentrations of H_2O_2 were used to induce cell death in primary osteocytes isolated from chick. Confirming the apoptotic nature of the response to H_2O_2, we demonstrated that the cell death was caspase-dependent since the isatin sulfonamide based selective inhibitor of caspase 3/7 inhibited H_2O_2 induced death.

Addition of 17β-estradiol at near physiological concentrations, shown to be non-toxic to the osteocyte cells, resulted in a total blockade of H_2O_2 induced apoptosis reducing levels to that of control cultures. Similarly both Raloxifene and LY117018 blocked H_2O_2 induced apoptosis in these cells. Interestingly previous studies have demonstrated that estrogen is capable of saving osteocytes and other bone cells from apoptosis induced by other chemical agents including dexamethasone [34] and etoposide [30]. In the case of dexamethasone induced apoptosis estrogen was working through an ER-dependent mechanism [34] and in the case of etoposide induced apoptosis estrogen was also shown to be working through the estrogen receptor via a non-genotropic signaling mechanism involving activation of Src/Shc/ERK pathway[30]. In our model system studied here, ERK activation does not appear to be an absolute requirement for estrogens ability to inhibit oxidant induced apoptosis since 17β-estradiol and SERMs retained their anti-apoptotic activity in the presence of the ERK1/2 inhibitor UO126 at concentrations known to block ERK phosphorylation in the MLO-Y4 cell type [13]. Interestingly, we have previously demonstrated that ERKs may also be involved in pro-apoptotic pathways in osteocytes since dexamethasone induced osteocyte apoptosis is associated with a transient increase in ERK1/2 phosphorylation which can be blocked by the ERK inhibitor UO126 [13].

With regard to the possible estrogen receptor-mediated nature of this response, pre-incubation of cells with the estrogen receptor antagonist ICI 182 780 did not inhibit the 17β-estradiol saving effect. The failure of ICI 182 780 to block the estrogen saving effects might at first inspection suggest a lack of receptor mediation in the response. However, conclusions are complicated by the fact that incubation of cells with the estrogen antagonist ICI 182 780 alone also prevented H_2O_2 induced apoptosis. For this reason, we also used the stereoisomer 17α-estradiol that has specific non-genomic activity [35], and in ER replete MLO-Y4 17α-estradiol was capable of similar levels of apoptosis blockade as 17β-estradiol pointing to a lack of involvement of the classical ER genomic pathways in the response. Furthermore the ability of estradiol and both SERMs to inhibit oxidant induced apoptosis in HEK293 cell line which are ER-α and ER-β negative [32,33] indicated an estrogen receptor-independent mechanism of apoptosis blockade in cells under oxidant attack. These findings concur with those in neuronal cell types in which ER-independent estradiol blockades of oxidant induced apoptosis have been reported [36].

In the absence of any evidence that both estrogen and SERMs are working through the estrogen receptor, it is important to consider alternative mechanisms of anti-apoptotic activity capable of blocking this oxidant induced apoptosis in osteocytes. Insight as to the anti-apoptotic nature of estrogen in this model system may be gained through consideration of the structure of the molecules used. The

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prevention of oxidative stress induced cell death in neurons by estrogen is proposed to be through its direct antioxidant activity and is related to the presence of the C3-OH grouping on the steroid A ring [20]. Our study also suggests that this moiety might be important in the ability of estrogen and related compounds to block oxidant induced cell death in osteocytes. Estrogen derivatives Mestranol and Quinestrol, which have a -methyl group and a -pentyl group respectively in the steroid A ring C3-OH position, did not prevent H_2O_2 induced apoptosis in osteocytes while the derivative 17α-ethinylestradiol which has a steroid C3-OH group but in all other aspects is structurally identical to Mestranol and Quinestrol, was capable of preventing H_2O_2 induced apoptosis. ICI 182 780 is a 7 alpha-alkylamide analogue of 17β-estradiol with the same antioxidant associated steroidal moiety. For this reason, it is possible that ICI 182 780 is also capable of antioxidant activity, a characteristic that would explain its ability to save osteocytes against oxidant attack in the absence of estradiol.

The SERMs Raloxifene and LY117018 were also capable of saving osteocytes from oxidant induced cell death. While these SERMs are not closely structurally related to estrogens, the hydroxyl group associated with the phenolic moiety of the SERMs is in principle capable of mimicking the steroid A-ring phenolic hydroxyl of 17β-estradiol. In line with the hypothesis that the hydroxyl moiety is responsible for antioxidant activity future work will establish more clearly the precise molecular design of SERMs required to counter this characteristic. In support of our hypothesis that the anti-apoptotic activity of both estrogen and SERMs could be associated with a possible direct free radical effect, all of the compounds tested in this study apart from Mestranol and Quinestrol were also able to reduce reactive oxygen species generation in osteocytes as detected by H_2DCF-DA.

While the controlled production of ROS has beneficial effects in host defense mechanisms, the accumulation of ROS in the aged can contribute to the pathogenesis of age associated disease [17]. Previous evidence suggests that oxidative stress might play a role in the etiology of osteoporosis with an association between increased levels of oxidative stress and reduced BMD [37]. ROS generation has also been shown to be important in the process of bone resorption being both produced by actively resorbing osteoclasts [38] and involved in the recruitment and activation of osteoclasts both *in vitro* and *in vivo* [39]. Experiments in rats *in vivo* have demonstrated that estrogen loss (OVX) lead to a reduced ability in osteoclasts to neutralize oxidants using antioxidant enzymes [40,41].

Our results demonstrate that osteocytes are sensitive to ROS levels and this poses questions related to the conditions under which osteocytes would be exposed to ROS *in vivo*. Hypoxia generates cellular ROS in a range of cell types and leads to both positive and negative cell behaviors. For example, hypoxia engendered ROS production is known to be part of the signaling response of the contractile process in cardiomyocytes [42]. Interestingly osteocyte hypoxia has been shown to occur in bone as a result of disuse with the suggestion that this forms part of a novel mechanotransduction pathway [43]. In addition, the ROS generator Nitric Oxide is known to be an important mechanotransduction related signal molecule in osteocytes [44]. Therefore the production and sustained exposure of osteocytes to ROS could result from active local generation as a result of active resorption, conditions of disuse induced hypoxia, mechanical load engendered cell signaling or following local inflammatory responses. The presence of estrogen and related compounds might by virtue of their antioxidant activities protect osteocytes from attack by ROS in the pre-menopausal or HRT/SERM treated individual. Our results demonstrate that at least in part the mechanism by which estrogen maintains a viable osteocyte population is due to its antioxidant activity. This study highlights the importance of understanding all aspects of estrogen activity in bone. It also points to the possibility that SERMs mimic not only the bone sparing activity but also the antioxidant nature of estrogens and related compounds. These data will contribute to our understanding of the molecular design criteria required in the production of estrogen replacement compounds with activities consistent with the maintenance of both bone mass and bone quality.

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